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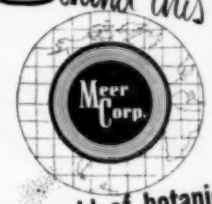

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JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

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Investigation of Stability in Emulsions of Varying Viscosities*

By EDWIN L. KNOECHEL† and DALE E. WURSTER

An investigation dealing with the possible effect of viscosity on the stability of oil-in-water emulsions is presented. Interpretation of the size frequency data on the basis of the volume-surface and weight-mean diameters indicated the possible presence of subvisual globules in the formulations. This possibility was not evident when the arithmetic-mean diameter was used. Experimental evidence indicated that viscosity played only a minor role in the gross stability of the systems studied.

THE FACTORS influencing emulsion stability have been the subject of many investigations. Thus, the influence of the type of emulsifying agent and its concentration (1–15), the density differential (1–4, 7, 14), and changes in environmental conditions such as those caused by elevated temperature (5, 11, 13) and centrifugation (7) have all been studied. The influence of methods of preparation on stability (1–3, 6, 9, 10, 12, 15) and the effect of globule size and distribution (16, 17) and other factors (15, 18) on the rheological flow properties of emulsions have also been investigated.

Few if any of the literature references deal specifically with the effect that changing the viscosity of the continuous phase or the viscosity of the emulsion might have on stability. Therefore, a series of emulsions of varying viscosities have been subjected to study.

METHOD OF STUDY

A stable emulsion has been described as one in which no sedimentation or creaming and no aggregation, flocculation, or coalescence of the discontinuous phase occurs. Since it has been pointed out previously that creaming decreases the interparticle distance, which can then lead to possible changes in the flocculation and coalescence rates, it would appear that the rates of all the above phenomena are interrelated (19). If the change in interparticle distance does cause significant variations in the rate of coalescence, then any existing relationship found between the coalescence and creaming rates would be most useful information.

Since the interparticle distances are related to the concentration and the particle size of discontinuous phase and the creaming rate, it is of interest to determine whether or not the rate of coalescence can be altered by changing the viscosity of the continuous phase.

Therefore, as a method of study, a series of emulsions of varying viscosities were prepared according to a standard procedure. Every attempt was made to hold all the variables, other than the viscosity of the continuous phase, constant. A size frequency analysis was employed to follow the changes that occurred within the emulsions. Because of the various methods employed in the literature to interpret the internal changes in aged emulsions (7, 11–13, 20) all the collected data were analyzed on the basis of the following values, the arithmetic-mean diameter [$d_{me} = \Sigma n_i d_i / \Sigma n_i$], the interfacial area [$Sd_{me} = 6/d_{me}$], the volume-surface mean diameter [$d_{vs} = \Sigma n_i d_i^3 / \Sigma n_i d_i^2$], the specific interfacial area [$S_{d_{vs}} = 6/d_{vs}$], and the weight-mean diameter [$d_{wm} = \Sigma n_i d_i^4 / \Sigma n_i d_i^3$], where, in all cases, n_i = the number of globules having a class diameter of d_i (12, 20–22). Also, the emulsions were followed for a longer period of time than reported by most workers.

* Received April 25, 1958, from the School of Pharmacy, University of Wisconsin, Madison.
Presented to the Scientific Section A. PH. A., Los Angeles meeting, April 1958.
The paper is based on a dissertation submitted by Edwin L. Knoechel to the Graduate School of the University of Wisconsin in partial fulfillment of the requirements for the degree of Doctor of Philosophy.
† Fellow of the American Foundation for Pharmaceutical Education.

EXPERIMENTAL

Preparation of Emulsions.—Large volumes of solutions of the various grades of methylcellulose, U. S. P. (10, 25, 50, 100, and 400 c. p. s.) were prepared with boiling distilled water, cooled, and stored at 5° for eight days to allow complete hydration. The solutions were then subjected to pressure filtration (Alsop "Sealed-Disc" filter, No. 51 filter pads) and finally stored at 25°. Microscopic examination of the resulting solutions indicated that they were free of particulate matter that might be confused with oil globules.

A solution of sodium lauryl sulfate, U. S. P. (10% w/v), was prepared with hot distilled water and cooled. Filtration through a medium porosity sintered glass funnel removed a brown sediment leaving the resulting solution clear and free of particles.

Emulsions (O/W) of varying viscosities containing 25% (v/v) liquid petrolatum, heavy, U. S. P., and 0.375% (w/v) sodium lauryl sulfate were formulated from the above solutions. Four 1-L. batches of each viscosity were prepared by mixing in a Waring Blendor for five minutes. The individual batches of the same viscosity were then pooled, mixed, and passed twice through a Tri-Homo colloid mill (rotor-stator setting 0.002 in.). Thirty sample bottles of each emulsion, each containing 100 ml. were stored at constant temperature (25°). No samples used in the subsequent analytical procedures were returned to storage for reuse later.

Viscosities of both the solution comprising the continuous phase and the complete emulsion were followed throughout the entire period of study. All viscosities were calculated from rheograms obtained using an Epprecht Rheometer (Drage Products, Inc.) at 25°.

The emulsions are referred to by number according to the viscosity grade of the methylcellulose used in the preparation of their external phases (i. e., 10, 25, 50, etc.).

Method of Analysis.—Each stored sample selected for analysis was first uniformly mixed with a specially constructed apparatus. The bottle was rotated at the rate of 10 r. p. m. for a total of 400 revolutions in a vertical plane in such a manner that it described a circle having a radius of 10 cm. After each 50 revolutions, the bottle was rotated 45° in the holder. With this treatment the discontinuous phase was evenly distributed. Size frequency determinations before and after this procedure indicated that no changes had occurred.

Dilution of the emulsion was accomplished by placing 2 to 7 drops into 25 ml. of distilled water in a glass-stoppered cylinder immersed in an ice-water bath. Twenty-five ml. of propylene glycol, U. S. P., was then added.

The size frequency analysis used to follow the changes that occurred in the emulsions employed a modified microscopic technique to classify the globules (23). A portion of the previously diluted emulsion was placed in a Bright Line hemocytometer (Am. Optical Co.) and viewed with a microscope (B. and L. Labrascope) equipped with a 97× oil immersion objective, a 20× ocular, and an Exton Euscope (B. and L.) (24). A special grid in which each scale division corresponded to 1 μ was attached to the Euscope screen. A carbon arc lamp fitted with a mechanical clock feed and a Pyrex water cell provided the illumination. A counting field was defined on this grid and the etchings of the hemocytometer were utilized in adapting a standard procedure so that no area was recounted. A period of fifteen minutes was allowed to elapse after charging the chambers before readings were taken. Over 100 fields were measured in each sample and three samples from separate bottles were employed for each determination. Over 4,000 globules were therefore counted in each analysis.

The size of each observed globule was recorded with a tape recorder and later transcribed onto data sheets. Globules in the visual range which measured up to one division were classified as having a diameter of 0.5 μ, while globules having a size larger than one but smaller than two divisions were classified as having a diameter of 1.5 μ, and so on.

From the size frequency data collected the d_{me}, Sd_{me}, d_{vs}, Sd_{vs}, and d_{vm} values were obtained with the aid of a magnetic-drum-data processing machine (type 650—IBM).[1]

The emulsions were analyzed at thirty-day intervals over a period of seven months.

RESULTS AND DISCUSSION

In Fig. 1, the per cent of globules of each size class (0.5 to 5.5 μ) is plotted against the aging time for the least viscous (no. 10) of the emulsions studied. Similar graphs were also obtained for the more viscous emulsions when the data contained in Table I was plotted. This type of plot shows very clearly the changes occurring in the systems. Thus, the major changes appeared to occur in the smaller size classes (0.5 to 2.5 μ).

The arithmetic-mean diameter (d_{me}) increased with time in all emulsions (Fig. 2).[2] The decrease in the corresponding interfacial area (Sd_{me}) with time is shown for the various emulsions in Fig. 3. The above effects were of course anticipated, but it is interesting to note that these emulsions of widely varying viscosities (34 to 1,800 c. p. s., see Table II) all behaved in a similar fashion. These data seem to indicate that the apparent rates of coalescence did

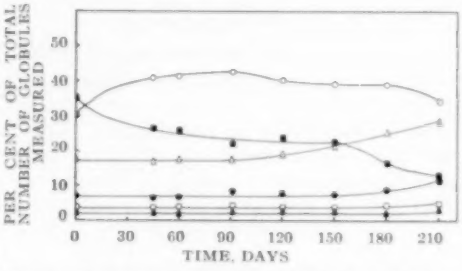

Fig. 1.—Per cent of 0.5–5.5 μ globules *vs.* time for emulsion number 10. ■ 0.5 μ; ○ 1.5 μ; △ 2.5 μ; ● 3.5 μ; □ 4.5 μ; ▲ 5.5 μ.

[1] The authors wish to thank the University of Wisconsin Numerical Analysis Laboratory for the use of this equipment and its facilities.

[2] Data for emulsion numbers 50 and 400 have not been plotted in Figs. 2 and 3 in order to make it easier to distinguish between the individual graphs of these figures.

TABLE I.—PER CENT OF THE TOTAL NUMBER OF GLOBULES MEASURED IN EACH SIZE CLASS IN THE VARIOUS EMULSIONS

Emulsion 10

Diameter, Microns	Time, Days							
	0	45	60	91	120	150	180	210
0.5	35.21	26.37	25.73	21.98	22.55	22.43	16.45	12.89
1.5	30.24	40.53	41.02	42.10	59.59	38.54	28.45	33.87
2.5	17.69	16.69	17.04	17.32	18.73	21.02	24.87	27.88
3.5	6.69	6.14	6.57	8.06	7.51	7.18	8.42	11.01
4.5	3.75	3.78	3.78	4.21	3.73	3.60	4.31	4.82
5.5	2.10	2.14	1.71	2.32	2.58	2.23	1.98	2.79
6.5	1.32	1.94	1.48	1.39	1.72	1.95	2.11	2.36
7.5	1.62	1.26	1.27	1.39	1.36	1.66	1.83	1.89
8.5	0.84	0.70	0.83	0.72	0.72	0.84	0.89	1.33
9.5	0.36	0.46	0.44	0.36	0.34	0.44	0.50	0.84
10.5	0.14	...	0.14	0.13	0.09	0.07	0.11	0.19
11.5	...	...	...	0.02	0.07	0.04	0.04	0.08
12.5	...	...	...	...	...	...	0.02	0.04

Emulsion 25

	Time, Days							
	0	30	60	90	120	149	180	210
0.5	40.27	38.10	30.90	30.80	24.51	22.71	20.72	18.10
1.5	32.48	37.04	41.39	43.14	46.62	41.22	40.88	39.19
2.5	15.96	13.10	15.73	16.06	18.04	21.41	24.73	25.63
3.5	6.18	6.72	7.35	5.96	6.74	8.50	7.57	9.74
4.5	3.15	3.79	3.27	3.18	3.01	4.41	3.92	4.66
5.5	1.33	1.16	1.08	0.75	0.94	1.54	1.74	1.91
6.5	0.20	0.10	0.24	0.14	0.14	0.18	0.45	0.71
7.5	0.03	...	0.04	...	...	0.02	...	0.06
8.5	0.03	...	...	...	...	...	...	...

Emulsion 50

	Time, Days							
	0	30	60	90	119	150	180	210
0.5	41.45	41.17	34.40	30.73	26.52	24.62	24.01	23.31
1.5	35.29	34.31	43.36	42.76	43.23	41.75	40.93	39.37
2.5	15.61	15.52	14.06	17.51	19.51	21.58	23.78	23.89
3.5	6.27	7.09	6.50	7.25	8.64	9.28	8.39	10.07
4.5	1.34	1.79	1.60	1.73	2.05	2.69	2.75	3.13
5.5	0.03	0.11	...	0.02	0.07	0.09	0.14	0.22

Emulsion 100

	Time, Days							
	0	30	60	90	120	150	181	210
0.5	46.26	41.45	36.89	29.02	30.23	25.53	24.84	20.75
1.5	28.05	31.95	34.72	40.08	42.54	41.16	37.58	37.22
2.5	15.89	17.11	16.83	20.09	17.88	19.09	25.51	26.15
3.5	9.05	8.61	10.58	9.60	8.46	11.80	9.90	12.57
4.5	0.75	0.89	1.00	1.21	0.88	2.43	2.17	3.24
5.5	...	...	...	...	...	...	0.02	0.06

Emulsion 200

	Time, Days							
	0	30	60	91	119	150	180	210
0.5	39.22	36.56	34.69	28.56	22.02	24.32	24.24	19.50
1.5	31.59	35.32	39.47	41.17	44.89	37.38	36.35	37.41
2.5	21.42	21.49	19.82	22.25	23.73	26.28	26.69	26.65
3.5	7.43	6.24	5.78	7.68	8.78	10.89	11.35	13.92
4.5	0.32	0.39	0.24	0.34	0.60	1.12	1.30	2.41
5.5	...	...	...	...	...	...	0.07	0.14

Emulsion 400

	Time, Days							
	0	31	60	90	120	150	181	210
0.5	39.70	34.13	30.92	28.23	25.84	24.84	19.50	14.76
1.5	31.16	37.81	40.47	42.53	42.13	37.97	37.62	37.56
2.5	25.20	24.16	24.18	25.31	26.41	28.42	32.37	34.16
3.5	3.56	3.56	4.16	3.67	5.36	8.33	9.60	12.23
4.5	0.37	0.34	0.27	0.26	0.27	0.39	0.83	1.16
5.5	...	...	...	...	...	0.07	0.09	0.12

not differ greatly. In fact the terminal value of the d_{me} was approximately 1.3 times larger than the initial value in all cases.

From the plots of the volume-surface mean diameter (d_{vs}) and the related specific interfacial area (Sd_{vs}) *versus* time (Figs. 4 and 5) it appears this diameter decreased and the Sd_{vs} increased during the first ninety to one hundred twenty days. After this time period the specific interfacial area decreased in nearly a linear manner with time (2, 3, 6, 13, 25).

TABLE II.—VISCOSITY OF THE AGED EMULSIONS AND METHYLCELLULOSE SOLUTIONS AT 25°

Fluid Number		Time, Days							
		0	30	60	90	120	150	180	210
10	ηe[a]	34	34	33	32	32	32	32	31
	ηcp[b]	9.5	9.0	9.0	9.0	9.0	9.0	9.0	9.0
25	ηe	95	94	92	92	90	91	91	89
	ηcp	20	20	20	20	20	20	20	19
50	ηe	205	204	202	205	203	202	198	198
	ηcp	35	36	35	35	35	34	34	34
100	ηe	460	450	445	440	436	432	430	428
	ηcp	80	80	79	79	79	78	79	78
200	ηe	830	830	828	844	836	820	812	812
	ηcp	130	130	130	129	128	125	122	120
400	ηe	1,800	1,790	1,775	1,770	1,765	1,755	1,740	1,740
	ηcp	270	270	260	262	262	262	260	260

[a] ηe = Viscosity (c. p. s.) of the emulsion.
[b] ηcp = Viscosity (c. p. s.) of the continuous phase (methylcellulose solution).

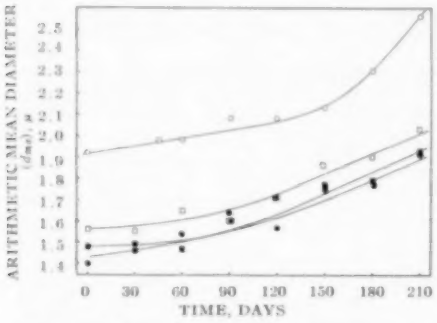

Fig. 2.—Arithmetic mean diameter, d_{ma}, *vs.* time for emulsions of varying viscosities. Emulsion number: ○ 10; □ 25; ● 100; ■ 200.

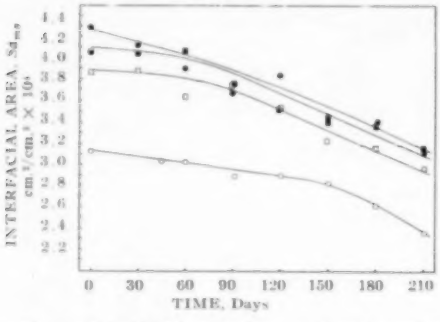

Fig. 3.—Interfacial area, $S_{d_{ma}}$, *vs.* time for emulsions of varying viscosities. Emulsion number: ○ 10; □ 25; ● 100; ■ 200.

A plot of the weight-mean diameter (d_{wm}) *versus* time (Fig. 6) shows a similar effect. From these data it would appear that the emulsions were becoming more instead of less stable during the first few time periods. This effect could be explained if it were assumed that subvisual globules coalesce in time to form visible globules. Other workers (11, 12) have indicated subvisual globules may be present; however, no reference was found in the literature where the possible effects of these particles are treated.

If the above interpretation is valid the initial values of d_{mc}, d_{vs}, and d_{wm} would be smaller and the interfacial areas larger. This serves to emphasize

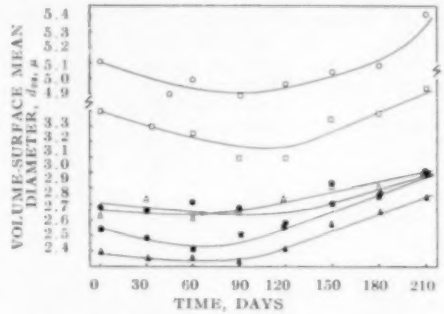

Fig. 4.—Volume-surface mean diameter, d_{vs}, *vs.* time for emulsions of varying viscosities. Emulsion number: ○ 10; □ 25; △ 50; ● 100; ■ 200; ▲ 400.

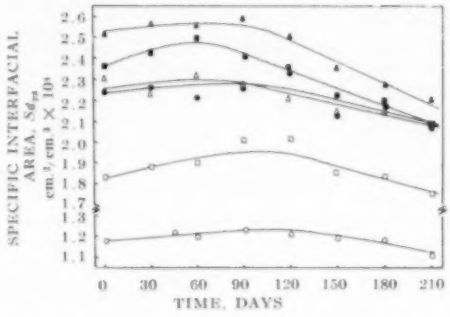

Fig. 5.—Specific interfacial area, $S_{d_{vs}}$, *vs.* time for emulsions of varying viscosities. Emulsion number: ○ 10; □ 25; △ 50; ● 100; ■ 200; ▲ 400.

one of the recognized limitations of the microscopic method of analysis. Thus, when this analytical method is employed with relatively stable emulsions, it is well not to rely upon a single value for interpretation of the data. Furthermore, the studies should be carried on for an adequate period of time. For example, if the investigation of these emulsions had been conducted for only three months the d_{vs} value would have shown just a decrease, and the d_{wm} value would have indicated almost no change, whereas the d_{mc} value shows an increase throughout. The d_{mc} value is predominantly a number distribution value

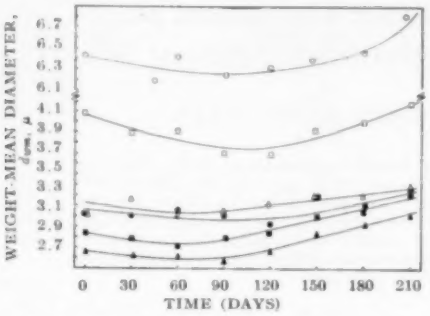

Fig. 6.—Weight-mean diameter, d_{wm}, *vs.* time for emulsions of varying viscosities. Emulsion number: ○ 10; □ 25; △ 50; ● 100; ■ 200; ▲ 400.

which is not appreciably affected by a small change in the number of large size globules, while the d_{vs} and d_{wm} factors are essentially area and volume distributions and are more influenced by the loss or gain of a few large globules (12). These inherent differences are more obvious when all the values are calculated. The voluminous amount of collected data could not have been processed in the various ways without the aid of the electronic computing equipment.

Table II shows that there was no appreciable variation in the viscosities of the emulsions or the methylcellulose solutions which constituted the continuous phase.

As mentioned previously, there appeared to be no marked differences in the rate of coalescence within the viscosity range of the tested emulsions for the duration of the study. Thus although interparticle distances may be assumed to decrease when creaming occurs, an increase in viscosity appeared to play only a minor role in the gross stability. Indeed, even the emulsion with the lowest viscosity appeared to be very stable. Other workers (4, 18) have suggested that viscosity is not of major importance. Whether changes in the interfacial film, the increased viscosity of the creamed layer, or other factors played a part were not determined in this study.

SUMMARY

A modified microscopic method was employed in a size frequency analysis study to follow the stability of a series of emulsions prepared by varying the viscosity of the continuous phase.

A numerical analysis method which made possible the interpretation of the data on the basis of the arithmetic, volume-surface, and weight-mean diameters was employed.

Experimental evidence indicating the presence of subvisual particles which can lead to unreliable diameters and interfacial area values during the early period of aging was obtained.

Only a slight variation in the apparent stability of the emulsions studied for a period of seven months was observed. Changing the viscosity did not seem to yield corresponding changes in the rates of coalescence. Thus, viscosity appeared to play only a minor role in the overall stability of these systems.

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Pentylenetetrazol Seizure Threshold in Meprobamate- and Phenaglycodol-Treated Mice*

By LINCOLN CHIN and EWART A. SWINYARD

The pentylenetetrazol infusion technique was tested for usefulness in the detection of tolerance and withdrawal hyperexcitability induced in mice by the chronic administration of meprobamate (Miltown, Equanil) and phenaglycodol (Ultran). The data obtained by this graded response procedure are remarkably similar to those previously obtained by a quantal electroshock method and suggest that the chemoshock technique may prove useful as a routine procedure for screening certain drugs for physical dependence liability.

THE WELL-KNOWN side effects and potential "behavioral toxicity" of the so-called "tranquilizing agents" justify concern over their indiscriminate use. The added possibility that these drugs might also induce physical dependence, as suggested by the original clinical reports of Barsa and Kline (1) and Lemere (2), stimulated the authors to study the effects of the chronic administration of these agents on the excitability of the central nervous system of mice. It was demonstrated by means of the experimental design of McQuarrie and Fingl (3) that tolerance and withdrawal hyperexcitability are induced by the chronic administration of meprobamate (4). Subsequent work (5) indicated that similar phenomena were induced by the chronic administration of phenaglycodol. On the basis of this experience and of additional clinical reports which indicate that physical dependence to meprobamate can be induced in man (6–12), it was suggested that this technique might prove useful as a presumptive test for screening candidate drugs for physical dependence liability (5). Although preliminary data are in agreement with this suggestion, the predictive value of this test can only be determined after more clinical and laboratory results have been accumulated on a larger number of centrally active drugs. Since this procedure utilizes a quantal end point, a relatively large number of animals is required for each determination; consequently, it is time-consuming and does not lend itself well to routine screening. Therefore, it was thought important to investigate a graded-response type of test in order to minimize the total number of animals required and, hence, to reduce the work involved. Because minimal seizures induced by the intravenous infusion of pentylenetetrazol closely resemble those induced by low-frequency electroshock, this chemoshock procedure was used in a study of meprobamate and phenaglycodol. The results obtained provide the basis for this report.

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METHODS

Male albino mice (Carworth Farms, CF #1 strain) weighing between 21.0 and 34.5 Gm. were employed as test animals. Since the primary objective of the study was to determine if the pentylenetetrazol infusion technique could be used to measure tolerance and withdrawal hyperexcitability, dose levels of meprobamate[1] and phenaglycodol[2] which are known to produce tolerance and withdrawal hyperexcitability (4, 5) were employed; the drugs were administered orally as a 2 or 3% suspension in 6% acacia solution.

Chemoshock Procedures.—Pentylenetetrazol seizures were induced by the technique of Orloff, *et al.* (13), as modified by McQuarrie and Fingl (14). The convulsant solution, composed of 0.5% pentylenetetrazol, 0.9% sodium chloride and 10 mcg./ml. of heparin sodium,[3] was infused into the tail vein at a constant rate of 0.005 ml. per second by means of the apparatus designed by Gabardi and Esplin (15), until the mouse exhibited persistent clonus for three seconds. The body weight and the pentylenetetrazol infusion time (the time in seconds from the start of the infusion until the end point was observed) were recorded for each animal. The data obtained for groups of drug-treated and control mice were evaluated by analysis of covariance (16), and the pentylenetetrazol seizure threshold ratio (threshold of test group divided by the threshold of control group) was calculated.

Chronic Administration of Meprobamate.—Twenty-six mice were randomly divided into two equal groups. Meprobamate was initially given to one group of mice in a total daily dose of 1,200 mg./Kg. (200 mg./Kg. at 8 a.m., 12 noon, 4 p.m., 8 p.m., and 400 mg./Kg. at midnight) for 7 days. On the eighth day each dose was increased by 50% to make a total daily dose of 1,800 mg./Kg.; this larger daily dosage was continued for seven days as indicated in Fig. 1. The other group of mice served

[1] Kindly supplied by Dr. F. M. Berger, Wallace Laboratories.
[2] Kindly supplied by Dr. K. K. Chen, Eli Lilly Co.
[3] Kindly supplied by The Upjohn Co.

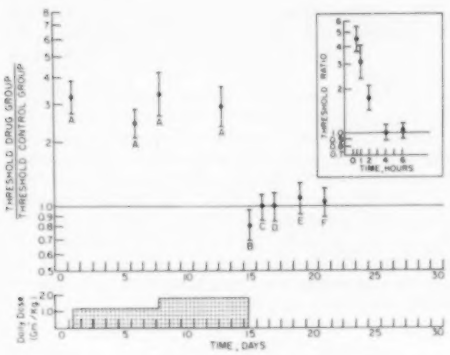

Fig. 1.—Effects of chronic administration of meprobamate (Miltown, Equanil) on pentylenetetrazol seizure threshold. Time in days is indicated on the abscissa. Pentylenetetrazol seizure threshold ratio (threshold for drug group/threshold for control group) is shown on the upper ordinate, and total daily dose of meprobamate is shown on the lower ordinate. Vertical bracketed lines indicate 95% fiducial limits. *A*, ratio one-half hour after 8 a. m. dose; *B*, *C*, *D*, *E*, and *F*, ratios at four, twenty-eight, fifty-two, one hundred, and one-hundred fifty hours, respectively, after the final dose of drug. The inset illustrates the duration (hours) of the effect of a single dose of meprobamate (300 mg./Kg.) on pentylenetetrazol seizure threshold in nontolerant mice.

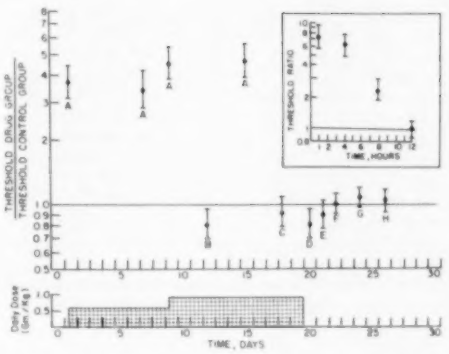

Fig. 2.—Effects of chronic administration of phenaglycodol (Ultran) on pentylenetetrazol seizure threshold. Time in days is indicated on the abscissa. Pentylenetetrazol seizure threshold ratio (threshold for drug group/threshold for control group) is shown on the upper ordinate, and total daily dose of phenaglycodol is shown on the lower ordinate. Vertical bracketed lines indicate 95% fiducial limits. *A*, ratio one hour after 8 a. m. dose; *B* and *C*, ratios eight hours after the midnight dose; *D*, *E*, *F*, *G*, and *H*, ratios at twelve, thirty-six, sixty, one hundred eight, and one hundred fifty-six hours, respectively, after the final dose of drug. The inset illustrates the duration (hours) of the effect of a single dose of phenaglycodol (300 mg./Kg.) on pentylenetetrazol seizure threshold in nontolerant mice.

as a control and was given the requisite volume of 6% acacia solution at the appropriate intervals. Pentylenetetrazol seizure threshold ratios were determined at the times indicated in the legend to Fig. 1.

Acute Administration of Meprobamate.—Forty-eight mice were randomly divided into four equal groups. Two groups were given orally a single dose of 300 mg./Kg. of meprobamate, and the other two groups the requisite volume of acacia solution. Pentylenetetrazol seizure threshold ratios were determined at selected times after drug administration. This procedure was repeated at intervals of six to ten days until threshold ratios were established one-half, one, two, four, and six hours after drug administration.

Chronic Administration of Phenaglycodol.—Thirty mice were randomly divided into two equal groups. Phenaglycodol was initially given to one group in a total daily dose of 600 mg./Kg. (200 mg./Kg. at 8 a.m., 4 p.m., and 12 midnight) for eight days. On the ninth day each dose was increased by 50% to provide a total daily dose of 900 mg./Kg.; this larger dosage was continued for nine days, as indicated in Fig. 2. The other group served as a control and was given the requisite volume of acacia solution at the appropriate intervals. Pentylenetetrazol seizure threshold ratios were determined at the times indicated in the legend to Fig. 2.

Acute Administration of Phenaglycodol.—Forty-eight mice were divided into four equal groups. Two groups were given orally a single dose of 300 mg./Kg. of phenaglycodol and the other two groups the requisite volume of 6% acacia solution. Pentylenetetrazol seizure threshold ratios were determined at selected times after drug treatment. This procedure was repeated at intervals of six to eight days until ratios were obtained one, four, eight, and twelve hours after drug administration.

RESULTS

Meprobamate-Treated Mice.—The administration of a single 300 mg./Kg. dose of meprobamate to nontolerant animals increased the pentylenetetrazol seizure threshold 4.5-fold one half hour after treatment (see inset, Fig. 1), whereas, after chronic treatment for seven days with 1,200 mg./Kg./day, and then for five days with 1,800 mg./Kg./day, this same dose of drug increased the seizure threshold only 2.9-fold (see *A*, day 13, Fig. 1). Thus, tolerance develops to the threshold-raising effect of meprobamate. Figure 1 also shows that, four hours after the 8 a.m. dose on day 15, the pentylenetetrazol seizure threshold in chronically treated animals was only 0.82 that of control animals. Since the seizure threshold four hours after the administration of a single dose of 300 mg./Kg. to nontolerant animals was the same as that of control mice at the 4-hour interval (see inset, Fig. 1), the reduction in the seizure threshold observed in the chronically treated animals must be attributed to the abrupt withdrawal of meprobamate.

Phenaglycodol-Treated Mice.—As illustrated in Fig. 2, tolerance also develops to the pentylenetetrazol seizure threshold-raising effect of phenaglycodol. This was demonstrated by the fact that the seizure threshold was increased 7.4-fold one hour following the administration of a single 300 mg./Kg. dose of phenaglycodol to nontolerant mice (see inset, Fig. 2), whereas after chronic treatment for eight

days with 600 mg./Kg./day and then for six days with 900 mg./Kg./day this same dose of drug elevated the seizure threshold only 4.7-fold (see *A*, day 15, Fig. 2). Figure 2 also shows that eight hours after the midnight dose on day 12, and twelve hours after the midnight dose on day 20, the seizure threshold ratios in the chronically treated mice were only 0.82 (see *B* and *D*, Fig. 2). Since the seizure threshold ratio after the administration of a single dose of 300 mg./Kg. to nontolerant mice was still 2.3 at the eight-hour interval, and was normal at the twelve-hour interval (see inset, Fig. 2), the reduction in seizure threshold observed in the chronically treated animals must be attributed to the abrupt withdrawal of phenaglycodol.

DISCUSSION

The data presented indicate that the pentylenetetrazol infusion technique is capable of detecting the tolerance and withdrawal hyperexcitability induced by the chronic administration and subsequent abrupt termination of large doses of meprobamate or phenaglycodol. Tolerance was shown by a 30 and 36% reduction in the threshold-raising effect of meprobamate and phenaglycodol, respectively, in animals receiving these agents chronically. Withdrawal hyperexcitability was demonstrated by the observation that four hours after the final dose of meprobamate and twelve hours after the final dose of phenaglycodol, the pentylenetetrazol seizure threshold in chronically treated mice was only 0.82 that of control animals (see *B*, Fig. 1 and *D*, Fig. 2).

Since the dose schedules employed in the present study are similar to those employed in previous studies (4, 5) in which the low-frequency electroshock technique was used to measure central nervous system excitability, it is of interest to compare quantitatively the results obtained with the two procedures. With regard to tolerance as demonstrated by the electroshock procedure in animals treated chronically, previous work has shown that the threshold-raising effect of 300 mg./Kg. of meprobamate is reduced by at least 75% (4) and that of 300 mg./Kg. of phenaglycodol is reduced by 50% (5). In the present studies, the pentylenetetrazol seizure threshold-raising effect of 300 mg./Kg. of meprobamate or of phenaglycodol is reduced by approximately 33% in chronically treated animals. With regard to withdrawal effects as demonstrated by the electroshock method in animals treated chronically, hyperexcitability was detectable four and eight hours after withdrawal of meprobamate and subsided within twenty-eight hours (4); hyperexcitability was detectable eight and twelve hours after withdrawal of phenaglycodol and subsided within thirty-two hours (5). In the present chronic studies, hyperexcitability was detectable four hours after meprobamate withdrawal and subsided within twenty-eight hours; hyperexcitability was detectable twelve hours after withdrawal of phenaglycodol and subsided within thirty-six hours. Thus, it can be concluded that the chemoshock technique is as useful as the electroshock technique for the detection of tolerance and withdrawal hyperexcitability induced by the chronic administration of large doses of these drugs.

In view of the fewer animals required, without sacrifice of precision, and the similar profiles of action obtained for meprobamate and phenaglycodol by the two procedures (intravenous pentylenetetrazol and low-frequency electroshock), the graded-response type technique reported herein should lend itself to the routine screening of candidate drugs for physical dependence liability. It must be emphasized, however, that the predictive value of this test can be determined only after more laboratory and clinical results have been accumulated.

SUMMARY

Tolerance and withdrawal hyperexcitability, induced in male albino mice by the chronic administration of large doses of meprobamate (Miltown, Equanil) or of phenaglycodol (Ultran), were determined by a pentylenetetrazol infusion technique. The development of tolerance was shown by the fact that the threshold-raising effect of meprobamate and phenaglycodol was decreased 30 and 36%, respectively, in chronically treated mice. The occurrence of withdrawal hyperexcitability was shown by the fact that, four hours after the final dose of meprobamate and twelve hours after the final dose of phenaglycodol, the seizure threshold was reduced approximately 18%. The use of the pentylenetetrazol technique as a routine procedure for screening certain candidate drugs for physical dependence liability is discussed.

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Quantitative Evaluation *in Vitro* of Oxytocics*

By ARTHUR J. SILVERMAN

The classic Magnus measurement of isolated smooth muscle strip activity has been improved by the use of simple quantitative expressions. These permit one to equate the activities of untreated "control" strips to their subsequent activities upon administration of drugs. A feature of this method is that it allows comparison of activities of strips from different animals of the same species. Particular application has been made to the recording of oxytocic activity of drugs on the guinea pig estrus uterus. The parameters used are the contraction frequency, amplitude, and duration. Ratios are constructed between the values observed for the untreated tissue compared to those of the treated strip. Refrigeration may be used in prolonging the life of the strip for extended study.

THIS is a description of an experimental technique that can be applied to the screening of various suspected oxytocics. The animal of choice is the English guinea pig, which has been given 0.03 mg./Kg./day i. m. of γ-estradiol for a period of three to four days to simulate an estrus uterus (1).

The uterine horn is extirpated and placed into a tissue isolate bath, maintaining a temperature between 37–39° (2). When recording the activity on the kymograph, the uterine horn is allowed to acclimate to the new environment of the bath for a duration of thirty to sixty minutes. The classic Magnus method of measurement is used for isolated smooth muscle strip and the activity is recorded on a kymograph.

Before a discussion of the method of measurement for activity, several terms applicable to the descriptive terminology of reporting quantitative evaluation of oxytocic activity should be reviewed; *duration* is the amount of time that the uterine horn remains contracted, *amplitude* is the highest peak reached during contraction, and *frequency* is the number of contractions per unit time.

The technique involved in measuring the activity of the uterine strip follows.

Measurement of Amplitude.—The normal contraction of the strip is measured from base line to the peak of the highest contraction of normal activity. The same procedure is followed after a drug has been administered to the same strip and the highest contraction is measured again. The direct measurement is on the kymograph paper and is expressed in centimeters. The denominator is the height of normal contraction and the numerator is the height of contraction after the drug is administered. See Figs. 1 and 2.

$$\text{Ratio of amplitude} = \frac{\text{Height of contraction with drug (cm.)}}{\text{Height of contraction without drug (cm.)}}$$

Measurement of Duration.—The amount of time the normal strip remains contracted is measured from start to finish of the contraction. Again, the same procedure is followed after a drug has been administered to the same strip. The direct measurement is on the kymograph paper and is expressed in centimeters. The denominator is the length of normal contraction without the drug and the numerator is the length of contraction with the drug administered. See Figs. 1 and 2.

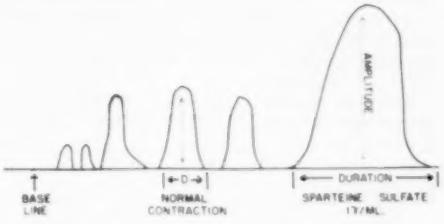

Fig. 1.—Effects of sparteine sulfate on a guinea pig estrus uterus. One mcg./ml.; amplitude, 13.6 cm./3.4 cm. = 4; duration, 3.52 cm./160 cm. = 2.2.

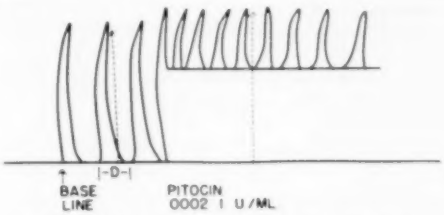

Fig. 2.—Effects of pitocin on a guinea pig estrus uterus. Amplitude, 3.5 cm./3.3 cm. = 1.08; duration, 1.5 cm./2.0 cm. = 0.75; frequency (6/min.)/(2/min.) = 3. Change in base line, 1.6 cm./3.3 cm. × 100 = 48 per cent.

$$\text{Ratio of duration} = \frac{\text{Length}^1 \text{ of contraction with drug (cm.)}}{\text{Length}^1 \text{ of contraction without drug (cm.)}}$$

Frequency.—The number of contactions per minute measured on the kymograph paper in the normal strip, as compared to the number of contractions after the drug has been administered in the bath. The denominator is the number of contractions per minute without the drug, and the numerator is the number of contractions per minute after the drug has been administered.

$$\text{Ratio of frequency} = \frac{\text{Number of contractions with drug/minute}}{\text{Number of contractions without drug/minute}}$$

* Received May 27, 1958, from the Ortho Research Foundation, Raritan, N. J.
Presented at the Fourth Pan American Congress of Pharmacy and Biochemistry (Pharmacology Section), Washington, D. C.

[1] Length represents a measurement of the period of time the strip remains contracted.

Two reference standards were used for this experimental technique: Pitocin[2] and sparteine sulfate.

Pitocin.—When used in minute quantities (0.0002 I. U./ml. bath volume–0.0005 I. U./ml. bath volume), the desired effects for each dose level were obtained, but the uterus could not be used for further tests because of the lasting oxytocic effects, even after several washings. The results fell into the following range:

Pitocin	Amplitude	Duration	Frequency
0.0002	1.05	0.7	2
0.0005	1.27	1.15	4

The above results show a definite oxytocic activity at levels of 0.0002 I. U./ml. to 0.0005 I. U./ml., respectively. The aforementioned figures when compared to the normal strips show an inverse relationship between duration and frequency. At the above levels of Pitocin the duration is diminished and frequency is increased.

Sparteine Sulfate (3).—The dose level for this preparation having a marked increase in activity is 1 mcg./ml.

Sparteine sulfate, γ/ml.	Amplitude	Duration	Frequency
1	4.0	2.2	0.8

This preparation has a marked effect *in vitro* on the uterine horn at 1 γ/ml. The merits of the compounds as to *in vitro* and *in vivo* activity shall not be discussed in this paper. Noting the above figures there is an inverse relationship between duration and frequency again, the duration is increased and the frequency is decreased as compared to the normal strip; the amplitude as for all oxytocic agents is increased.

An extension of this experimental technique is to see how long the uterine horn shows activity *in vitro*. Recordings were observed for a period of eight days; the first four days very little change in the pattern was seen. During the next four days a gradual decrease was noted; by the end of the eighth day there was no activity. The temperature was the key factor in maintaining uterine motility. The following procedure was carried out to study eight day uterine movement: (*a*) The uterine horn was placed in the tissue isolate bath, containing Tyrode solution at 37–39°, with oxygen bubbled into the bath and activity recorded for eight hours. (*b*) At the end of eight hours the uterine horn was taken out along with its Tyrode solution and placed in the refrigerator at 4° for sixteen hours. (*c*) This procedure was repeated for eight days and recordings taken every day.

The reason for describing this procedure at this time is that it may be of interest in the study of uterine activity, and the possibility of carrying out the screening procedures for long periods of time on the same uterine horn. Results may then be compared to the original records of the same normal untreated uterine horn.

[2] Pitocin is the registered trademark of Park, Davis and Co.

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Derivatives of 8-Hydroxy-2-quinoline Acrylic Acid*

By MADHUKAR G. VAIDYA,† and JOSEPH G. CANNON

8-Hydroxyquinaldine and its alkyl ethers have been utilized to prepare a new series of 2-quinoline acrylic acids and their ethyl esters. These compounds have been synthesized as possible antifungal and/or antibacterial agents, and as intermediate compounds for further synthetic studies.

One of the most significant moieties in chemotherapy is the quinoline ring system. The fact that many of the physiologically active alkaloids contain this nucleus has given great impetus to attempts to synthesize (among other compounds) better anti-infective agents derived from quinoline. 8-Aminoalkylaminoquinoline derivatives such as pentaquine and pamaquine are potent antimalarials; 8-quinolinol has been shown to possess antifungal (1–4) and antibacterial (5, 6) properties. 8-Ethoxyquinoline has shown promising antibacterial activity (5, 7); 8-hydroxyquinaldine has antibacterial activity (7); 2-styryl quinolines have been shown to possess antibacterial and trypanocidal activity (8–10). Halogenated 8-hydroxyquinaldines have been incorporated into soaps as antiseptic agents (11). 2-Quinoline acrylic acid has been demonstrated to have some tuberculostatic activity (12).

It was felt, therefore, that a quinoline molecule containing a combination of the groups shown to enhance the chemotherapeutic activity of the quinoline ring might well possess interesting biological properties. Ethers of 8-hydroxy-2-quinoline acrylic acid contain an oxygen function at position 8 and an unsaturated side chain at

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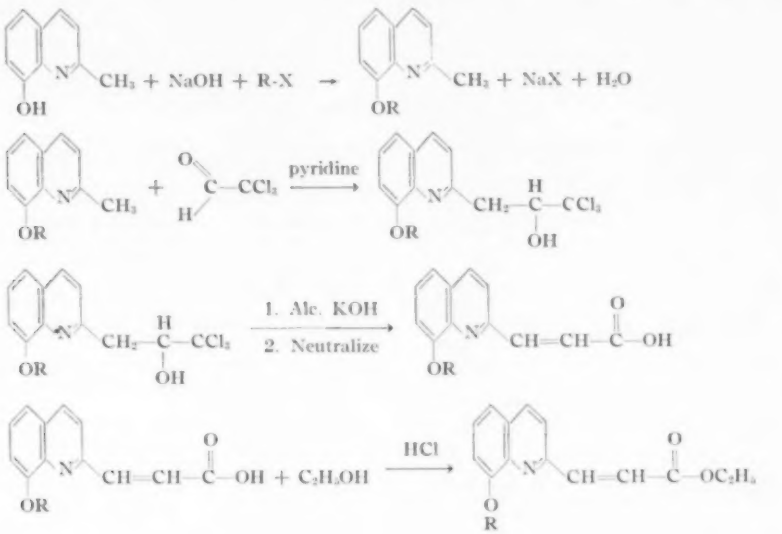
position 2, terminating with a carboxyl group, all of which have been demonstrated to increase physiologic activity.

The compounds reported herein were prepared for testing as antibacterial and antifungal agents. Certain derivatives of them should also be amenable to further elaboration of the molecule; this will be the subject of a future communication.

The general route used for the synthesis was as indicated:



EXPERIMENTAL

8-Hydroxyquinaldine.—This compound was prepared in 20% yield by the method of Doebner and von Miller (13), m. p. (uncorrected) 72–74°. (Doebner and von Miller reported a melting point of 74°.)

Ethers of 8-Hydroxyquinaldine.—*Method A* was identical with that used for the preparation of 8-hydroxyquinaldine (14).

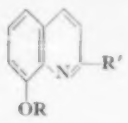
Method B.—Eleven grams (0.069 mole) of 8-hydroxyquinaldine was dissolved in 50 ml. of absolute ethanol in a 250-ml. standard-taper round-bottom flask equipped with a reflux condenser and a calcium chloride tube. To this solution was added 4 Gm. (0.1 mole) of sodium hydroxide dissolved in 4 ml. of distilled water. To the resulting mass 0.1 mole of alkyl halide was added, and the mixture was refluxed on a steam bath for five hours. The alcohol was then evaporated on a steam bath at atmospheric pressure. The gummy residue was dissolved in about 200 ml. of water and this solution was extracted several times with ether. The combined ethereal extracts were washed with a 100-ml. portion of 2.5% sodium hydroxide solution, and then with water. The ether extract was dried over anhydrous sodium sulfate, the ether was removed on a steam bath, and the oily residue was distilled under reduced pressure or crystallized. (See Table I.)

Method C.—The operations were similar to those used in method B, except that 0.2 mole of sodium hydroxide and 0.1 mole of alkylaminoalkyl chloride hydrochloride were used, and the reflux period was twenty hours. The crude heterocyclic ether thus obtained was purified by distillation under reduced pressure. (See Table I.)

Condensation of 8-Hydroxyquinaldine and Its Ethers with Chloral.—Chloral was prepared from chloral hydrate by distilling over sulfuric acid (15). To 0.1 mole of 8-hydroxyquinaldine or its ether contained in a 100-ml. standard-taper round-bottom flask equipped with a reflux condenser and a calcium chloride tube was added 15 ml. of pyridine (dried over potassium hydroxide pellets) and 0.1 mole of chloral, and the mixture was heated on a steam bath for two hours. The resulting tarry reaction mixture was poured into about 250 ml. of ether, the ethereal solution was filtered, and the ether was removed on a steam bath. An excess of 1:6 hydrochloric acid was added to the residue and the resulting solution was heated to boiling. It was then filtered to remove tars, and the filtrate was cooled. On neutralizing the filtrate with solid sodium carbonate, γ,γ,γ-trichloro-α-(8-hydroxy- or alkoxy-2-quinolyl)-β-hydroxypropane precipitated from solution, and was recrystallized from ethanol. (See Table I.)

8-Hydroxy-2-quinoline Acrylic Acid.—*Method D.*—Twenty-five hundredths of a mole of potassium hydroxide was dissolved in 70 ml. of absolute ethanol in a 500-ml. standard-taper round-bottom flask equipped with a reflux condenser and a calcium chloride tube. To this, 0.05 mole of γ,γ,γ-trichloro-α-(8-hydroxy-2-quinolyl)-β-hydroxypropane dissolved in 50 ml. of absolute ethanol was added. The mixture was heated very cautiously on a water bath until the violent reaction subsided, then it was refluxed on a steam bath for five hours. The alcohol was evaporated on a steam bath at atmos-

TABLE I.—DERIVATIVES OF 8-HYDROXY QUINALDINE



No.	R	R′	Method of Preparation	Yield, %	M. P. or B.P.[b]	Analyses[c]
1	H	CH_2—CHOH—CCl_3	...	67.6	113–114°	Calcd. for $C_{12}H_{10}Cl_3NO_2$: C, 47.1; H, 3.27; N, 4.57. Found: C, 47.4; H, 3.62; N, 4.58.
	...	Picrate[a]	...	...	190–192°, decompn. without melting	Calcd. for $C_{18}H_{13}Cl_3N_4O_9$: N, 10.45. Found: N, 10.65.
2	H	CH—CH—COOH	D	25.6	205–206° with decompn.	Calcd. for $C_{12}H_9NO_3$: C, 66.99; H, 4.19; N, 6.51. Found: C, 66.81; H, 4.44; N, 6.5.
3	H	CH—CH—$COOC_2H_5$	...	75.6	85–86°	Calcd. for $C_{14}H_{13}NO_3$: C, 69.2; H, 5.35; N, 5.76. Found: C, 69.35; H, 5.75; N, 5.72.
4	CH_3	CH_3	A[d]	20.3	124–125°	...
5	CH_3	CH_2—CH(OH)—CCl_3	...	79.2	127–128°	Calcd. for $C_{13}H_{12}Cl_3NO_2$: C, 48.64; H, 3.74; N, 4.36. Found: C, 48.56; H, 3.92; N, 4.32.
	...	Picrate	...	...	198–200° with decompn.	Calcd. for $C_{19}H_{15}Cl_3N_4O_9$: N, 10.2. Found: N, 10.42.
6	CH_3	CH—CH—COOH	E	27.8	197–198° with decompn.	Calcd for $C_{13}H_{11}NO_3$: C, 68.13; H, 4.79; N, 6.11. Found: C, 68.25; H, 4.75; N, 6.30.
7	CH_3	CH—CH—$COOC_2H_5$	...	74.2	51–52°	Calcd. for $C_{15}H_{15}NO_3$: C, 70.01; H, 5.84; N, 5.45. Found: C, 70.2; H, 5.62; N, 5.51.
8	CH_2—CH_3	CH_3	B	77.2	52–53°	Calcd. for $C_{12}H_{13}NO$: C, 77.00; H, 6.95; N, 7.48. Found: C, 76.8; H, 6.85; N, 7.7.
	...	Picrate	...	...	194–196°	Calcd. for $C_{18}H_{16}N_4O_8$: N, 13.45. Found: N, 13.15.
9	CH_2—CH_3	CH_2—CH(OH)—CCl_3	...	85.2	146–147°	Calcd. for $C_{14}H_{14}Cl_3NO_2$: C, 50.20; H, 4.18; N, 4.18. Found: C, 50.00; H, 4.02; N, 4.41.
	...	Picrate	...	...	182–183°	Calcd. for $C_{20}H_{17}Cl_3N_4O_9$: N, 9.95. Found: N, 9.65.
10	CH_2—CH_3	CH—CH—COOH	E	32.2	214–216° with decompn.	Calcd. for $C_{14}H_{13}NO_3$: C, 69.20; H, 5.35; N, 5.76 Found: C, 69.5; H, 5.32; N, 5.82.
11	CH_2—CH_3	CH—CH—$COOC_2H_5$	...	80.7	104–105°	Calcd. for $C_{16}H_{17}NO_3$: C, 70.8; H, 6.27; N, 5.17. Found: C, 70.6; H, 6.45; N, 5.17.
12	CH_2—CH_2—CH_3	CH_3	B	74.6	108–110°/ 0.2 mm.	Calcd. for $C_{13}H_{13}NO$: C, 77.7; H, 7.47; N, 6.95. Found: C, 77.3; H, 7.25; N, 7.27.
	...	Picrate	...	...	225–227°	Calcd. for $C_{19}H_{16}N_4O_8$: N, 13.02. Found: N, 13.4.
13	CH_2—CH_2—CH_3	CH_2—CH(OH)—CCl_3	...	69.2	100–101°	Calcd. for $C_{15}H_{16}Cl_3NO_2$: C, 51.7; H, 4.6; N, 4.02. Found: C, 57.5; H, 4.62; N, 3.8.
	...	Picrate	...	...	152–153°	Calcd. for $C_{21}H_{19}Cl_3N_4O_9$: N, 9.7. Found: N, 9.82.
14	CH_2—CH_2—CH_3	CH—CH—COOH	E	24.8	181–182°	Calcd. for $C_{15}H_{15}NO_3$: C, 70.01; H, 5.83; N, 5.45. Found: C, 70.20; H, 5.65; N, 5.72.
15	CH_2—CH_2—CH_3	CH=CH—$COOC_2H_5$	...	76.2	81–82°	Calcd. for $C_{17}H_{19}NO_3$: C, 71.6; H, 6.66; N, 4.92. Found: C, 71.36; H, 7.03; N, 5.05.

TABLE I. (*Continued*)

16	$CH_2—CH_2—N—(C_2H_5)_2$	CH_3	C	43.7	140–142°/0.4 mm.	Calcd. for $C_{16}H_{22}N_2O$: C, 74.5; H, 8.15; N, 10.85. Found: C, 74.52; H, 8.45; N, 10.75.
	.....	Picrate[a]	...	...	163°	Analytically pure. Sample could not be obtained.
17	$CH_2—CH_2—N—(C_2H_5)_2$	$CH_2—CH(OH)—CCl_3$	...	52.5	83–84°	Calcd. for $C_{18}H_{25}Cl_3N_2O_3$: C, 53.3; H, 5.68; N, 6.92. Found: C, 53.75; H, 5.72; N, 6.81.
18	$CH_2—CH_2—N—(C_2H_5)_2$	$CH=CH—COOH$	E	21.2	198–200° with decompn.	Calcd. for $C_{18}H_{22}N_2O_2$: C, 68.2; H, 6.96; N, 8.92. Found: C, 68.32; H, 6.82; N, 9.09.
19	$CH_2—CH_2—N—(C_2H_5)_2$	$CH=CH—COOC_2H_5$	...	77.6	75–76°	Calcd. for $C_{20}H_{26}N_2O_2$: C, 70.2; H, 7.6; N, 8.19. Found: C, 70.36; H, 7.47; N, 8.34.

[a] All picrates recrystallized from ethanol.
[b] All m. p.'s or b. p.'s uncorrected.
[c] All analyses by Drs. Weiler and Strauss, Oxford, England.
[d] This compound was prepared by Doebner and von Miller (14).

pheric pressure and the residue was extracted with a convenient amount of water. This aqueous extract was filtered to remove the undissolved tarry matter, and the clear filtrate was made faintly acidic to Congo red paper by the addition of glacial acetic acid. The precipitated heterocyclic acid was collected on a filter and was recrystallized from ethanol. (See Table I.)

8-Alkoxy-2-quinoline Acrylic Acids.—*Method E.*—A procedure similar to the one used for 8-hydroxy-2-quinoline acrylic acid was employed, except that 0.2 mole of potassium hydroxide was used. (See Table I.)

Ethyl Esters of Acids.—Five thousandths mole of the acrylic acid was dissolved in 25 ml. of absolute ethanol in a 250-ml. standard-taper round-bottom flask equipped with a reflux condenser and a calcium chloride tube, and the solution was saturated with dry hydrogen chloride gas. It was then refluxed on a water bath for two hours. The solution was evaporated to dryness on a steam bath, and the gummy residue was dissolved in a convenient amount of water. This aqueous solution was neutralized with solid sodium bicarbonate, and it was extracted with ether. The ether was removed from the extract on a steam bath, and the oily residue was crystallized from Skelly B. (See Table I.)

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A Colorimetric Assay for the Determination of Diethylstilbestrol*

By J. D. DUERR and B. A. PAPPAS

A procedure is described whereby the concentration of diethylstilbestrol can be determined in pharmaceutical preparations. The color developed upon the addition of sulfuric acid and ferric chloride to alcoholic solutions of diethylstilbestrol is proportional to the concentration of diethylstilbestrol, but only over a small range. Samples of commercial implant pellets containing diethylstilbestrol were analyzed and the results tabulated.

APART from its importance in human therapy, diethylstilbestrol, during recent years, has been used in increasing quantities in fattening farm animals prior to marketing.

In 1931 Kober (1) discovered that when estrin is heated with concentrated sulfuric acid and phenol it gives rise to a yellow color. The addition of water and reheating produces a specific pink color. This reagent and modified versions have been used by Venning (2), Banes

* Received June 9, 1958, from Chas. Pfizer & Co., Inc., Process Development Laboratory, Pharmaceutical Production and Packaging Department, Brooklyn, N. Y.

(3), and Haenni (4) for the determination of estrogenic ketosteroids. The factors affecting the stability and intensity of the color developed in the Kober reaction according to Venning are: the composition and the amount of reagent used, length of time during which the sample is heated to develop the immediate color, the amount of water added to the yellow mixture, and the time of reheating required to convert the yellow color to the pink color.

Haenni (4) showed that the reaction is influenced by the ferric ion. He described a set of stable Kober reagents containing appreciable amounts of ferric salt and water to obviate the effects of those substances.

In 1946 Devis (5) reported the reactions of diethylstilbestrol in chloroform with concentrated sulfuric acid and various oxidizing agents. He claimed that when a water-free-chloroform solution of diethylstilbestrol was added to the following oxidizing agents in concentrated sulfuric acid the corresponding colors were produced in the chloroform phase: chromic oxide and potassium dichromate gave red colors; antimony pentachloride, bismuth chloride, and titanium sulfate gave red colors; selenium oxide and molybdenum oxide gave red or violet colors; and uranium oxide nitrate or acetate gave red-orange colors.

When aqueous ferric chloride was added to the diethylstilbestrol in organic solvent no color was produced because the diethylstilbestrol was insoluble in the water.

Both sulfuric acid and ferric chloride have been used for the identification of diethylstilbestrol (6). Concentrated sulfuric acid forms an orange color and ferric chloride forms a green color which changes to yellow.

The principle involved in the official U. S. P. assay is as follows: After preparation of the sample and extraction of the diethylstilbestrol into ether and then alcohol, aliquots of the alcoholic solution are evaporated to dryness and treated with a reagent containing 100 mg. vanadyl sulfate and 20 ml. of sulfuric acid brought to 200 ml. with glacial acetic acid. The mixture is then heated on the steam bath for one hour, cooled, and the absorbance read at 520 mμ against a similarly prepared blank.

It was discovered, however, that when a mixture of concentrated sulfuric acid and aqueous ferric chloride was added to an alcoholic solution of diethylstilbestrol an intense violet color was produced. The violet color was found to be proportional to the concentration of diethylstilbestrol present. Further investigation showed that the relationship holds true to Beer's law only through a limited range. It was, however, sufficiently large to permit accurate determinations.

The reagent proposed here, for the assay of diethylstilbestrol resembles the Kober-Haenni reagent in that it contains concentrated sulfuric acid and a ferric salt. The phenol, hydrogen peroxide, and water have been eliminated and the length of time for the preparation of the reagent has been cut from twenty-four hours to about fifteen minutes. Furthermore, the method proposed does not involve heating the sample other than that produced by the addition of the reagent to the ethanol solution.

EXPERIMENTAL

Absorption Maximum.—A sample of pure diethylstilbestrol was dissolved in 3A alcohol to give a concentration of approximately 500 μg./ml. The reagent composed of concentrated sulfuric acid and ferric chloride was prepared as follows: Forty milliliters of concentrated sulfuric acid were added to 1 ml. of an aqueous solution of 10% ferric chloride hexahydrate. The reagent was then covered and allowed to cool to room temperature before use. A 3-ml. aliquot containing approximately 1.5 mg. of diethylstilbestrol was transferred to a 25-ml. volumetric flask. The aliquot was then diluted to 5 ml. with 3A alcohol. Five milliliters of 3A alcohol were used for the blank. To the aliquot and blank were added 4 ml. of the ferric chloride–sulfuric acid reagent. The reagent was added in 1-ml. portions with constant swirling to avoid spattering. The solutions were then allowed to stand in water at 20° for five minutes. 3A alcohol was then carefully added almost to the mark. The solutions were shaken vigorously and replaced in cold water. The color was allowed to develop for ten minutes. At the end of five minutes the flasks were brought to the mark, shaken, and replaced in cold water. At the end of the full ten minutes the active sample was read against the prepared blank on a Cary Recording Spectrophotometer. The absorption maximum was found to be at 558 mμ. The graph is shown in Fig. 1. A secondary peak was found at 420 mμ but was not related to concentration of diethylstilbestrol. This procedure was followed several times and the absorption maximum was always found to be at 558 mμ. The absorption values in each case were the same for equal concentrations of diethylstilbestrol.

Conformity with Beer's Law.—A solution of diethylstilbestrol in 3A alcohol was prepared to contain exactly 500 μg./ml. Aliquots containing 0.50, 1.00, 1.50, 2.00, and 2.50 mg. were transferred with pipets to glass-stoppered 25-ml. volumetric flasks. The aliquots were diluted to 5 ml. with 3A alcohol. Five milliliters of 3A alcohol were used for the blank. The procedure used in the determination of the absorption maximum was followed. The absorbances of the aliquots were read at 558 mμ against the prepared blank. The absorbances obtained were plotted against their respective concentration per aliquot. It was found that concentrations from 0 to

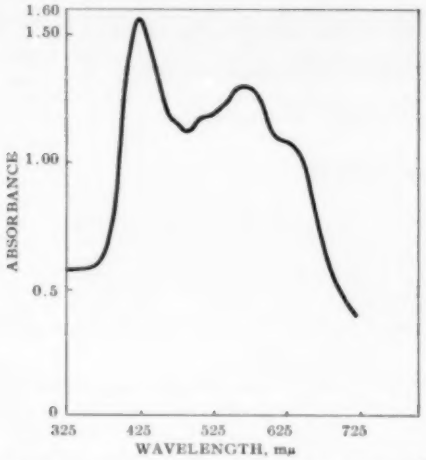

Fig. 1.—Absorption spectrum of diethylstilbestrol and ferric chloride, sulfuric acid reagent in alcoholic solution. Determined on Cary Recording Spectrophotometer Model 11 using 2-cm. cells.

1.0 mg. per aliquot and over 2.0 mg. per aliquot did not follow Beer's Law. However, aliquots containing between 1.0 and 2.0 mg. showed that the relationship between concentration and absorbance followed Beer's law. These points were then plotted and the straight line obtained was used as a reference for the determination of diethylstilbestrol in all subsequent assays.

Assay of Commercially Available Pellets.—Commercially available implant pellets containing 12 mg. of diethylstilbestrol were assayed in the following manner: Twenty pellets were weighed and the average weight was determined. The pellets were then ground to a fine powder in a mortar and pestle. A sample of the resulting powder was shaken with enough ethyl alcohol to give a final concentration of 400 μg./ml. The solids remaining were then filtered off and 3, 3.5, and 4-ml. aliquots of the filtrate were transferred to 25-ml. glass-stoppered volumetric flasks. The aliquots were diluted to 5 ml. with ethyl alcohol and 5 ml. of ethyl alcohol was used for the blank. The same assay procedure was again followed as in the previous experiments. The results which are the average of three aliquots are shown in Table I. The internal variation is about 3%. Also shown in Table I are results obtained by L. N. Mattson of Scientific Associates of St. Louis, Mo., using the assay described in the U. S. P. XIV and D. R. Manley of Chas. Pfizer Analytical Research Laboratory using an ultraviolet radiation method (7).

DISCUSSION

It is indicated from the results obtained, that pharmaceutical products containing diethylstilbestrol, especially in tablet form, can be assayed using the procedure outlined, and the results compare favorably with those obtained using the official U. S. P. assay.

TABLE I.—ASSAY OF COMMERCIALLY AVAILABLE PELLETS[a]

Lot No.	Label	Assay	Recovery, %	Assay[b]	Assay[c]
094A	12	12.5	104	..	..
094B	12	12.0	100	..	..
095B	12	11.6	97	11.9	12.2
096A	12	11.7	98	..	..
096B	12	11.5	96	10.8	12.4
097A	12	11.7	98	12.7	12.6
097B	12	12.9	108	..	..
098	12	12.6	105	..	..
099	12	12.1	101	..	..
100	12	11.8	98	12.0	12.5
101	12	12.8	106	..	..
102	12	11.9	99	11.2	13
103	12	12.2	102	..	..
104	12	12.1	101	..	..
105	12	11.9	99	12.5	13
106	12	11.9	99	12.0	13.6
107	12	13.3	111	..	..
108	12	12.0	100	..	..
109	12	11.5	96	11.6	12.6
110	12	12.5	104	..	..
111	12	12.1	101	..	..
112	12	12.2	102	..	..
112R	12	13.5	112	..	..
113	12	12.0	100	..	..
114	12	12.2	102	..	..
115	12	11.8	98	11.9	12.4
116	12	11.9	99	..	..
117	12	12.0	100	12.5	12.6
118	12	12.5	104	..	..
119	12	11.6	97	..	..
121	12	12.4	103	..	..

[a] Assays reported in mg./pellet.
[b] Assayed by Leland N. Mattson of Scientific Associates, St. Louis, Mo.
[c] Assayed by D. R. Manley, Chas. Pfizer & Co. Inc., by an ultraviolet radiation method (7).

SUMMARY

1. Diethylstilbestrol reacts with concentrated sulfuric acid and ferric chloride to produce a violet color with an absorption maximum at 558 mμ.

2. Concentrations of diethylstilbestrol in pellet formulations have been accurately determined.

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The Sterilization of Polyethylene Bags by Electron Irradiation and a Bacterial Monitor as a Measure of Sterility*

By JOHN J. MAYERNIK and THOMAS DANIELS

By means of a bacterial monitor, it was found that a 2 megarad dose of high velocity electrons will sterilize 0.353 inch of polyethylene. A new bacterial monitor was devised which serves as a direct measure of the sterilizing effect of irradiation on microorganisms. The bacterial monitor is a sealed polyethylene envelope containing polyethylene strips infected with spores of *Bacillus thermoalimentophilus*. The bacterial monitor may be used to establish the sterilizing capabilities of electron accelerators and may be used as a routine control check on sterilization by irradiation.

BETA RAYS emitted by high voltage electron accelerators and gamma rays emitted by radioisotopes have been shown to be capable of sterilizing a wide variety of materials including foods, drugs, surgical supplies, and human tissues (1, 2, 3). However, the number of products actually sterilized by irradiation on a practical basis is very few. One of the severest restrictions of electron irradiation to application in sterilization is the limited depth of penetration of the electron beam. Dunn (4) and Duffey (5) reported that the ionization produced by an electron beam of 2-Mev. energy is adequate for most sterilization applications. The total penetrating range of a 2-Mev. electron beam has been shown by Foster (6) and Knowlton (7) to be roughly 0.4 inch in material of unit density, and that for each additional Mev. the depth of penetration is increased by 0.2 inch. However, only about 60% of the total penetrating depth is useful in sterilization (6). Accordingly, the sterilizing penetration of a 2-Mev. beam is estimated to be approximately 0.24 inch in material of unit density.

It was desired to develop polyethylene bags for use as sterile antibiotic containers. Since polyethylene is heat sensitive, it cannot be sterilized by conventional heat sterilization procedures and therefore electron irradiation was explored as a possible means of sterilization.

After it was determined that polyethylene can be sterilized by irradiation, it was desired to establish the thickness of polyethylene that could be sterilized by the particular accelerator to be used. In order to determine the completeness of sterilization at various thicknesses, it was decided to measure the effect of irradiation directly on microorganisms rather than to monitor the accelerator by chemical or physical devices. Microorganisms have been used by a number of workers (4, 8–10) to measure the bactericidal effect of beta and gamma rays either in the dry, liquid, or frozen states in either test tubes, Petri plates, or in ampuls. Since these techniques were inconvenient for our use, a new bacterial monitor was devised for measuring the sterilizing effect of irradiation on polyethylene bags.

The new bacterial monitor (Fig. 1) is a sealed polyethylene envelope containing polyethylene strips infected with dry spores of *Bacillus thermoalimentophilus*.[1] This organism was selected as the test organism since Dunn (4) found bacterial spore-forming organisms to be more resistant to the lethal action of electron irradiation than other microbes. In addition, studies in our laboratory showed *Bacillus thermoalimentophilus* to be more resistant to destruction by heat than one strain each of *Bacillus mycoides* and *Bacillus pumilus*, two strains of *Bacillus subtilis*, and three strains of *Bacillus cereus*.

In order to serve as a measure of sterilization, the bacterial monitor is placed under polyethylene bags so that during irradiation the electron beam must pass through the bags before passing through the monitor. After irradiation the monitor is tested and if it is found to be sterile it is concluded that the superimposed bags have been sterilized.

EXPERIMENTAL

Preparation of Bacterial Monitors.—Spores of *B. thermoalimentophilus* were obtained by growing the organism on nutrient agar plates at 33° for eight days. The spores were harvested, centrifuged, washed three times with isotonic sodium chloride solution, and then dried under vacuum at 60°. One gram of the dried material was found to contain 3×10^9 spores.

This stock supply of spores was used to infect polyethylene strips (2.0 x 0.5 x 0.002 inches) by dusting in a plastic bag (20 x 13 inches). It was considered desirable to infect each strip with

* Received May 24, 1958, from the Chemical Control Laboratories, Merck & Co., Inc., Rahway, N. J.

[1] Strain originally obtained from L. W. Charkey, Colorado Agricultural and Mechanical College, Fort Collins.

approximately 100,000 viable spores. This degree of infection was obtained experimentally by finding the weight of dried spores needed for the number of strips introduced into the dusting bag. After the spores and the strips were introduced into the dusting bag, it was partly inflated, heat sealed, and then shaken vigorously for about twenty minutes. Then the bag was opened, six sample strips were removed, and the number of viable spores per strip was determined by the following procedure. Each strip was placed into a test tube with 10 ml. of isotonic sodium chloride solution containing 1% Tween 20 and shaken vigorously to remove the spores from the strip. An aliquot of the suspension was then plated out in nutrient agar, and after incubation for two days at 33°, the number of colonies was counted.

A bacterial monitor was prepared according to the following method. Polyethylene sheeting of 0.002 inch thickness, measuring approximately 12 x 10 inches, was folded in half and the two ends were heat sealed by means of a Vertrod Impulse sealer to form an envelope measuring approximately 12 x 5 inches. By means of forceps, three infected strips were placed into the envelope and the fourth side of the envelope was heat sealed. The three strips in the envelope were separated from each other by two additional heat seals across the width of the envelope. Thus, a bacterial monitor (Fig. 1) was produced which measured only 0.006 inch at its thickest portion. The dimensions of the monitor may be modified to fit the size of the object being irradiated.

Sterilizing Penetration of Electron Beam Through Polyethylene.—The effective sterilizing penetration of a 2-Mev. electron beam through polyethylene (density 0.92) was determined by irradiating layers of polyethylene film of graded thicknesses together with their respective subjacent monitors and then testing the latter for surviving spores. The graded thicknesses of polyethylene were prepared by stacking 12 x 6 inch sheets of polyethylene of 0.006 inch thickness until the desired thickness was obtained. Each stack of polyethylene sheets was maintained intact by sealing it in a 13 x 7 inch polyethylene bag to form a packet. Preliminary irradiation tests of a wide range of polyethylene thicknesses indicated the sterilizing depth of penetration to be between 0.3 and 0.4 inch. Accordingly, packets were made of ten different thicknesses covering this range. The thickness of each packet was measured at ten different positions by means of a Cady automatic hand micrometer and the average thickness and tolerance interval of each packet was calculated. These measurements are recorded in Table I.

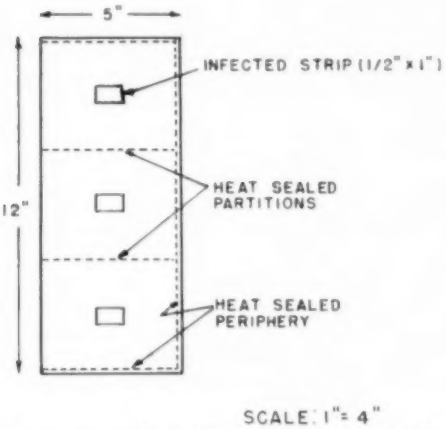

Fig. 1.—Bacterial monitor (infected strips within a polyethylene envelope.)

The packets were irradiated in the beam of a Van de Graaff accelerator[2] using a 2-Mev. energy and a beam current of 250 microamperes to produce a 2 megarad irradiation dose. The packets were irradiated on each of four separate days with intervals of at least one week between runs. In order to avoid bias due to any factor during an irradiation run, each packet was irradiated at random according to a preassigned random code number. Whenever a packet was irradiated a new monitor was placed under it and the monitor was given a number corresponding to that of the packet. Following irradiation the monitors were tested for sterility and for number of survivors by viable spore count. To determine sterility, the outside of each monitor was disinfected with 0.1% aqueous solution of benzalkonium chloride, the envelope aseptically opened and two strips each were transferred to individual test tubes containing 40 ml. of sterile thioglycollate broth. After incubation for seven days at 33° the tubes were examined for growth. The surviving number of viable spores was determined on the third strip by the procedure outlined previously under the preparation of bacterial monitors. The results of the sterility tests and of the viable spore counts are reported in Table I, and the relation between sterilization and polyethylene thickness is illustrated in Fig. 2.

DISCUSSION

The results in Table I and Fig. 2 show that polyethylene with a thickness of 0.353 inch or less was sterilized in every case by a single exposure to a 2 megarad dose of high velocity electrons. In the thickness range of 0.353–0.371 inch, one test strip out of twelve showed surviving organisms which indicates that a doubtful zone exists in this range in which sterilization is usually but not always accomplished. Polyethylene with a thickness of 0.371 inch or more was not sterilized in any case by a 2 megarad dose of electrons. The evidence presented in Table I can be used as a basis of establishing a routine sterilizing procedure for polyethylene bags which would provide an adequate margin of safety in sterilization.

It was mentioned earlier that Foster (6) and Knowlton (7) found the total penetrating range of a 2-Mev. electron beam in material of unit density to be 0.4 inch. Since only 60% of the total penetrating range was found useful for sterilization, the sterilizing depth of penetration was expected to be approximately 0.24 inch. In our studies with the bacterial monitor, the sterilizing depth of penetration of 0.353 inch of polyethylene at a density of 0.92 is equivalent to 0.32 inch of material at unit density. Accordingly, the bacterial monitor was useful in indicating that the sterilizing depth of penetration achieved by the 2-Mev. electron beam under our

[2] Irradiation was performed by High Voltage Engineering Corp., Burlington, Mass.

TABLE I.—RESULTS OF STERILITY TESTS AND OF VIABLE SPORE COUNTS OF TEST STRIPS AFTER IRRADIATION OF VARIOUS THICKNESSES OF POLYETHYLENE

Polyethylene Thickness, Average ± Tolerance Interval, Inches[a]	Sterility Test,[b] Growth per Strip				Viable Spore Count,[c] Number per Strip			
	Day 1	Day 2	Day 3	Day 4	Day 1	Day 2	Day 3	Day 4
0.298 ± 0.0057	− −	− −	− −	− −	0	0	0	0
0.314 ± 0.0069	− −	− −	− −	− −	0	0	0	0
0.332 ± 0.0086	− −	− −	− −	− −	0	0	0	0
0.341 ± 0.0093	− −	− −	− −	− −	0	0	0	0
0.356 ± 0.0084	− −	− −	− −	− −	0	0	0	0
0.362 ± 0.0093	− −	+ −	− −	− −	0	0	0	0
0.379 ± 0.0117	+ +	+ +	+ +	+ +	10	30	45	10
0.388 ± 0.0087	+ +	+ +	+ +	+ +	0	155	580	300
0.389 ± 0.0127	+ +	+ +	+ +	+ +	0	100	100	635
0.405 ± 0.0245	+ +	+ +	+ +	+ +	10	1,800	910	6,100

[a] Of the measurements taken over the area of the film, 95% are expected to fall within this interval.
[b] Key to symbols: − − = no growth in each of 2 strips, + − = growth in 1 strip and no growth in the other, + + = growth in each of 2 strips.
[c] Average preirradiation infection was 160,000 viable spores per strip.

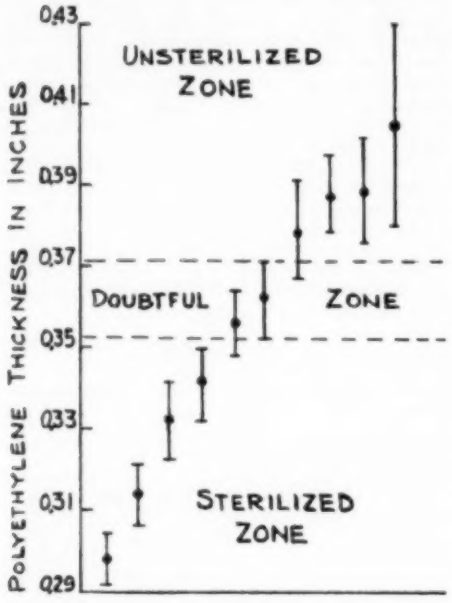

Fig. 2.—Relation between sterilization and polyethylene thickness (average with tolerance interval).

experimental conditions was one-third greater than theoretically expected.

The bacterial monitor was found to be a simple and effective means of confirming that polyethylene bags have been sterilized by irradiation and for establishing the thickness of polyethylene that a particular generator is capable of sterilizing. It can also be used as a positive system of control during routine sterilization by irradiation. It is superior to chemical or physical monitors in that it serves as a direct measure of the effect of irradiation on one of the most resistant types of organisms that one is attempting to destroy. The ease of preparation and testing of the monitor makes it a useful, adaptable, and practical tool in the field of radiation sterilization.

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A Preliminary Report of the Effect of Gastric Mucous upon Tablet Disintegration*

By D. D. ABBOTT, E. W. PACKMAN, E. W. REES, and J. W. E. HARRISSON

In vitro **disintegration tests have served as a control procedure in the production of uniform tableted products. These tests do not necessarily reflect the true *in vivo* disintegration of the tablet. A major factor which may be responsible is the effect of the thick, viscous, adhesive mucous which is constantly being produced within the alimentary tract. Disintegration values for a variety of tablets were determined in U. S. P. simulated gastric juice and in human gastric juice. In addition, these tablets were exposed to gastric mucous obtained from human subjects and then evaluated for disintegration properties in simulated gastric juice. Exposure to mucous for periods of three to five minutes significantly lengthened the disintegration time.**

THE *in vitro* TESTING of the disintegration time of tablets has been the subject of much debate and experimentation for many years. A review of the problems of such an *in vitro* determination and the methodology involved has been made by Vliet (1). Although the methods have been greatly standardized and refined over the past twenty years, it is recognized that these testing procedures do not and were not necessarily developed to duplicate conditions within the gastrointestinal tract. The testing procedures that have been and are presently being employed do, however, afford a check procedure in the manufacture of uniform tablets.

This present preliminary investigation was initiated under the stimulus of a recent study carried out in our laboratories. In this investigation employing several aspirin-containing analgesics (2), we observed varied absorption patterns from tablets exhibiting comparable *in vitro* disintegration times. Since Brodie, *et al.* (3), have shown that acetylsalicylic acid is absorbed from the stomach, we surmised that these competitive tablets may not have disintegrated in the stomach as uniformly as they did *in vitro*. This observation can possibly be explained by the fact that mucoid material present in gastric juice may, under certain conditions (i. e., swallowing a tablet dry without water), coat a tablet, thus rendering it more resistant and prolonging its disintegration time.

Based upon this premise, we have studied the *in vitro* disintegration of several commercial tablets in simulated gastric juice (U. S. P.) and in two samples of pooled human gastric juice. Each of the samples of human gastric juice contained a different concentration of mucoid substance. In addition, to simulate what could conceivably occur if a tablet was ingested without water, in the early morning, on an empty stomach, we determined the effect of immersing tablets in mucoid substance, separated from human gastric juice.

* Received April 25, 1958, from LaWall and Harrisson Research Laboratories, Philadelphia, Pa.

METHODS

Determination of Disintegration Time.—The "basket-rack apparatus" employing six tablets and the *in vitro* disintegration procedure described in the U. S. P. XV were employed in all of the tests. Disintegration of the tablets was evaluated in U. S. P. simulated gastric juice and two different samples of pooled human gastric juice. Human gastric juice was obtained by intubating hospitalized preoperative ulcer patients with a number 12 French Levine Tube and aspirating samples over a four-hour period. Each of the pooled samples of gastric juice employed in our study was made up of secretions collected over a three-day period from four adults. Approximately one gallon of human gastric juice was available for our tests.

Determination of Mucoidal Content of Gastric Juice.—An aliquot of gastric juice was placed into a centrifuge tube and allowed to stand for fifteen minutes. The upper foamy or purulent layer was discarded. After centrifuging for twenty-five minutes at 1,500 r. p. m., the supernatant clear gastric juice was decanted and the precipitate removed. The precipitate was then dried in a vacuum oven for four hours to a constant weight. Mucoidal content was expressed as milligrams per 100 ml. gastric juice. Sample A—human gastric juice contained 45 mg. dried mucoidal material per 100 ml. gastric juice and sample B—human gastric juice contained 102 mg. dried mucoidal material per 100 ml. gastric juice. This latter content of mucoid material is considerably lower than is often found in gastric juice.

Tablets Evaluated.—Tablets of acetylsalicylic acid; acetylsalicylic acid plus buffer; acetylsalicylic acid, acetophenetidin, and caffeine; dihydroxyaluminum sodium carbonate; sodium bicarbonate; calcium carbonate, magnesium carbonate, and magnesium trisilicate; and tablets of prochlorperazine were evaluated for their *in vitro* disintegration properties in the three "experimental solutions."

Direct Exposure of Tablets to Mucoidal Substance.—As an additional procedure, tablets from three of the tablet formulations were completely immersed in the wet mucoidal substance obtained from sample B gastric juice for five minutes. The

tablets were then removed and *in vitro* disintegration time determined in simulated gastric juice by the usual U. S. P. procedure.

RESULTS

Comparative disintegration times of the seven samples of tablets in simulated gastric juice, human gastric juice containing 45 mg. mucoidal substance per 100 ml. of juice, and human gastric juice containing 102 mg. mucoidal substance per 100 ml. juice are presented in Table I and Fig. 1.

In vitro disintegration in simulated gastric juice of tablets which had previously been exposed to mucoid material separated from gastric juice are presented in Table II.

Fig. 1.—Comparative disintegration times.——— simulated gastric juice,— —human gastric juice 45 ml. mucoid/100 ml., — – ———human gastric juice, 100 ml. mucoid/100 ml. *A*, acetylsalicylic acid; *B*, acetylsalicylic acid, buffered; *C*, acetylsalicylic acid, acetophenetidin, and caffeine; *D*, dihydroxy aluminum sodium carbonate; *E*, sodium bicarbonate; *F*, calcium carbonate, magnesium carbonate, and trisilicate; *G*, prochlorperazine, sugar coated.

DISCUSSION

It has been well established by Babkin (4) and many other investigators that the volume and constituents of gastric juice contained within the stomach at any one time are not constant. Variations may be dependent on the physical and/or mental condition of the patient, the presence or absence of stress, environmental conditions, and even the time of day. That these variations in volume and composition exist, cannot and should not be overlooked when one attempts to predict the extent to which a tablet will disintegrate in the stomach.

In vitro disintegration is prolonged when a tablet is exposed to "separated mucous" (Table II), a similar phenomenon may occur if a tablet is ingested in the early morning on an empty stomach. We have found that gastric juice collected from fasting patients or in the early morning often contains a large proportion of mucous and, therefore, the physiologic availability of the active agent could be decreased on a time basis.

Attempts to limit the *in vitro* disintegration of tablets at sixty minutes (5, 6, 7) or thereabouts, will not solve the problem completely since each tablet may react differently within the mucous-containing gastric juice of the stomach. Certainly if a given tablet disintegrates *in vitro* a few seconds before the "permissible time limit," we cannot expect the medicament to be as readily available for physiologic absorption as that in a tablet which may take only one-fourth or one-tenth the permissible time limit to disintegrate *in vitro* even though both tablets fall within the defined time limit.

If, as we have observed in this preliminary investigation, gastric mucous does alter the disintegration time, perhaps some suitable mucoid (mucoprotein) or some suitable thickener should be incorporated into the U. S. P. formulation to further simulate gastric juice. This might approximate more closely the mucous or viscous component of gastric secretion. Of course, it would be ideal to screen tablets in both simulated gastric juice and human gastric juice which contained a specific concentration of mucoid substance; this approach does not seem practical at the moment.

The data which we have presented may offer a partial explanation for the many heretofore unex-

TABLE I.—DISINTEGRATION TIME

Contents of Tablets	U. S. P. Simulated Gastric Juice	Human Gastric Juice—A, 45 mg. Mucoid/100 ml.	Human Gastric Juice—B, 102 mg. Mucoid/100 ml.
Acetylsalicylic acid	18–30 sec.	1–2 min.	3–8 min.
Acetylsalicylic acid plus buffer	25–40 sec.	1–3 min.	3–6 min.
Acetylsalicylic acid, acetophenetidin, and caffeine	2–4 min.	6–8 min.	9–13 min.
Dihydroxy aluminum sodium carbonate	13–15 min.	18–21 min.	29–34 min.
Sodium bicarbonate	29–31 min.	35–39 min.	45–54 min.
Calcium carbonate, magnesium carbonate, and magnesium trisilicate	12–16 min.	20–22 min.	28–32 min.
Prochlorperazine	7–12 min.	11–14 min.	19–24 min.

TABLE II.—EFFECT OF PREVIOUS EXPOSURE TO MUCOID MATERIAL UPON *in-Vitro* TABLET DISINTEGRATION

Contents of Tablets[a]	Disintegration Time, Simulated Gastric Juice—Before Exposure to Mucous	After Exposure to Mucous	Approximate Ratio
Acetylsalicylic acid	18–30 sec.	10–12 min.	1:24
Acetylsalicylic acid plus buffer	25–40 sec.	9–14 min.	1:40
Acetylsalicylic acid, acetophenetidin, and caffeine	2–4 min.	18–21 min.	1:5

[a]Tablets exposed to mucoid material from gastric juice B for five minutes prior to determining *in vitro* disintegration time in simulated gastric juice.

plainable variations encountered in the blood levels or absorption patterns of many drugs. The unpredictable effect of normal, as well as abnormal gastric secretions, upon tablet disintegration remains a factor that is not presently controlled by *in vitro* disintegration tests.

SUMMARY

1. We have determined *in vitro* disintegration time of several commercial tablets in simulated gastric juice and human gastric juice.

2. *In vitro* disintegration is prolonged in human gastric juice. There is some relationship between prolongation of disintegration and gastric mucoid content. A high mucoid content of gastric juice may double disintegration time and may increase it sixteen or more times.

3. *In vitro* disintegration is prolonged in simulated gastric juice if the tablets are first exposed to mucoidal material from human gastric juice.

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Study of the Cation Exchange Properties of Bentonite With Application to the Assay of Pharmaceuticals*

By WILLIAM G. GORMAN† and EARL P. GUTH

An acid activated bentonite fraction was exchange saturated with gentian violet, and this complex is used for the study of exchange reactions employing organic cations. The effects of time, temperature, pH, concentration, ratio of solution to exchanger, solvent, and the nature of the cation are studied. An assay procedure is proposed wherein the amount of dye released from an exchange complex would indicate the concentration of cation or organic solvent in solution. The exchange of organic cations is compared for a few different exchange materials.

As ONE OF THE FIRST used ion exchange materials, bentonite has been employed in a great number of diverse fields. Many studies have been conducted on its adsorption and exchange properties. In general, however, the studies on exchange were conducted on inorganic cations while the studies on adsorption were on organic cations. Although Gieseking (1) reported, in 1939, that large complex organic cations could be exchanged with other cations of approximately the same size and shape, little work appears to have been done on such organic exchanges. It was the object, therefore, of this investigation to study the exchange of organic cations on bentonite, and to explore the possibility of applying such exchange reactions to the assay of organic cations.

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EXPERIMENTAL

Preparation of Acid Activated Bentonite.—It was reported by Gonzalez and Garcia (2) and Gonzalez and Deitz (3) that acid treatment of bentonite increases the lattice spacing and the surface area. Lamar (4) and others found that acid activation increases the adsorption properties of bentonite. Thus, it was decided to use acid activated bentonite in this study.

One kilogram of bentonite[1] was added to 7.5 liters of distilled water in a large container and allowed to hydrate. One hundred fifty milliliters of concentrated sulfuric acid were incorporated and the mixture was allowed to macerate for twenty-four hours. Two liters of alcohol were added and the resultant mixture was centrifuged for ten minutes at a force of approximately 8,000 times gravity. Two distinct layers were observed in the sediment. A lower granular-appearing layer, B, and an upper lighter-colored, creamy-appearing layer, C, were separated by a thin, chocolate-colored line. The two layers were carefully separated, and each fraction was mixed with four liters of alcohol. The magmas were centrifuged and the resultant sediments were dried on a steam plate. The hard lumps resulting were ground with a ball mill and passed through a

[1] BC Volclay (American Colloid Company).

100-mesh sieve. This powder was washed with alcohol on a Büchner funnel until the washings were free of sulfate. The fractions were dried again and passed through a 100-mesh sieve. The weight of fraction B (bentonite-B) was approximately five times that of fraction C (bentonite-C).

Potentiometric titrations were performed on bentonite-B and C. Both titration curves followed the pattern of weak acids with pKa values of about 4.0. The curves also correspond to that of hydrogen bentonite as given by Coleman and Harward (5).

The exchange capacity of each fraction was determined using the method of Robertson and Ward (6). The values so obtained were 53.9 and 53.4 milliequivalents per 100 Gm. for bentonite-B and C, respectively. Due to the similar nature of the two fractions and the predominance of fraction B, bentonite-B was used in most of these studies.

Preparation of Gentian Violet Bentonite-B.—One hundred grams of bentonite-B were mixed with 16 Gm. of powdered gentian violet and 900 ml. of distilled water. The mixture was then dried on a steam plate with occasional stirring. The dried residue was powdered and washed three times with 900-ml. volumes of distilled water. The residue was dried again on a steam plate and passed through a 100-mesh sieve. The resulting purple-black powder will be referred to as gentian violet bentonite-B or GVB-B.

Montmorillonite can be saturated with any particular cation (7). Organic cations, if sufficiently hydrophilic, may be introduced into clays by ion exchange (8). It is also reported (8) that large cations, such as methylene blue, can quantitatively displace all exchangeable base from a clay. Since care was taken to insure saturation of the bentonite-B with the dye, GVB-B is believed to be bentonite-B with gentian violet as the principal exchangeable cation.

General Method Used to Measure the Extent of Exchange.—Unless otherwise specified, 25 ml. of the given drug solution were added to 200 mg. of GVB-B. The mixtures were then shaken and kept at 25 ± 0.5° for one hour, then filtered. The extent of exchange at equilibrium was determined by measuring the absorbance of the dye released, which was determined against a blank prepared in the same manner, but with distilled water substituted for the drug solution. The absorbances were determined either with a Fisher AC Model Electrophotometer with a 595 mμ filter and standard 23-ml. adsorption cells (FE), or with a Cenco-Sheard-Sanford Photelometer with 575 mμ filter and 1-cm. adsorption cells (CSSP).

Cation Exchange Rate.—The rate of an organic cation exchange reaction was studied for GVB-B and a 0.00367 *N* procaine hydrochloride solution. The general method was used except that the time of contact of GVB-B with the solution was varied. Table I shows the absorbances of the dye released (FE) after various times of contact. It can be seen that equilibrium was established rapidly and showed little change even after forty-eight hours of contact. The experimental results for the exchange of procaine for gentian violet from GVB-B are in agreement with inorganic exchanges observed by other workers (9–12).

TABLE I.—ABSORBANCE OF DYE RELEASED FROM GENTIAN VIOLET BENTONITE-B BY PROCAINE HYDROCHLORIDE SOLUTION FOR VARIOUS TIMES OF CONTACT

Time of Contact	Absorbance of Dye Released
10 min.	0.213
15 min.	0.211
20 min.	0.213
30 min.	0.212
48 hr.	0.214

Effect of Temperature.—The effect of temperature was studied on the exchange of procaine for gentian violet for a system of GVB-B and a 0.00367 *N* procaine hydrochloride solution. The general method was followed except that the mixtures were kept at 15.0, 25.0, 35.0, or 45.0°, with all temperatures ±0.5°, for one hour before filtering. The absorbances of the dye solutions at each temperature (FE) are shown in Fig. 1. The graph shows that the amount of dye removed by distilled water increased with an increase in temperature. Since water contains virtually no ions for exchange, it would appear most probable that the dye removed in this case was by desorption. The increase in the release of dye with an increase in temperature follows the pattern of desorption as reported by other workers (13, 14). The amount of dye removed by the procaine hydrochloride solution also increased with an increase in temperature. If the exchange reaction were not affected by temperature, the dye released by the procaine hydrochloride solution would still increase with an increase in temperature due to the desorption effect just described. Subtraction of the distilled water curve from the procaine hydrochloride curve gave the resultant dye release curve for the procaine hydrochloride solution. The fact that this curve has a small positive slope indicates that the exchange reaction increased slightly with an increase in temperature. This is in harmony with the reports of other workers (15–17) who found either little or no effect due to temperature in the exchange reactions they studied.

Ratio of Solution to Exchanger.—As pointed out by Nachod and Wood (16), exchange reactions are second order bimolecular reactions and, as such, should be affected by the ratio of solution to exchanger. The general method was followed for a system of GVB-B and a 0.00367 *N* procaine hydro-

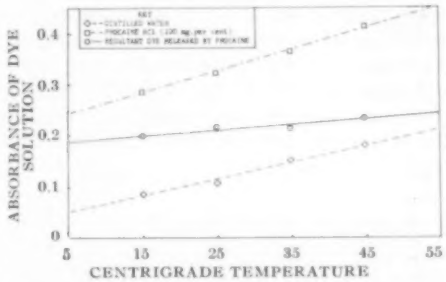

Fig. 1.—Effect of temperature on the release of dye from gentian violet bentonite-B by procaine hydrochloride solution.

chloride solution, except that various weights of GVB-B (50, 100, 150, 200, 250, and 500 mg.) were used. The absorbance of the dye released (FE) for each weight of GVB-B is shown in Fig. 2. The graph shows a marked increase in the absorbance of the dye released as the weight of the GVB-B was increased up to a weight of 150 mg. where a leveling off occurred. A plot of the absorbance of the dye released (FE) *versus* the ratio of solution to exchanger is shown in Fig. 3. A straight line of negative slope resulted showing that the concentration of the dye solution decreased with an increase in the solution to exchanger ratio even though the amount of dye released increased with the solution to exchanger ratio.

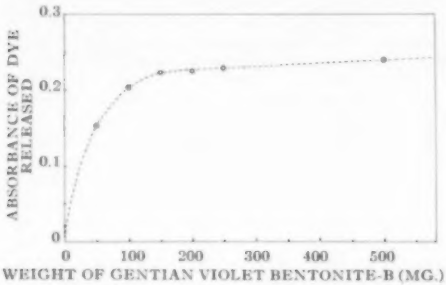

Fig. 2.—Absorbance of dye released from various weights of gentian violet bentonite-B by 25 ml. of procaine hydrochloride solution (100 mg. per cent).

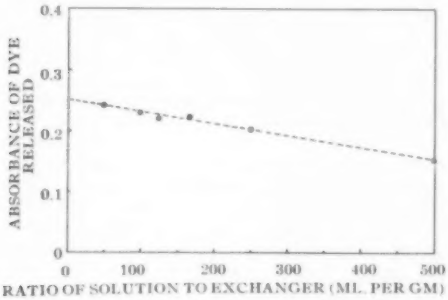

Fig. 3.—Absorbance of dye released from gentian violet bentonite-B by procaine hydrochloride solution for various ratios of solution to exchanger.

Effect of Solution Concentration.—The general exchange method was followed with various solutions (diphenhydramine hydrochloride, procaine hydrochloride, strychnine sulfate, and caffeine alkaloid) of different normalities (0.0025, 0.0050, and 0.0100 *N*). The absorbance of the dye released (CSSP) was plotted against the solution concentration in Fig. 4. The graph shows that the amount of dye released increased with the solution concentration, although not in direct proportion. However, a steep curve, such as that of diphenhydramine hydrochloride, should provide a simple and rapid means of estimating solution concentrations.

The entire procedure was repeated again using different concentrations of hydrochloric, sulfuric, and monochloroacetic acids. Due to the fact that gentian violet changes color in strongly acid media,

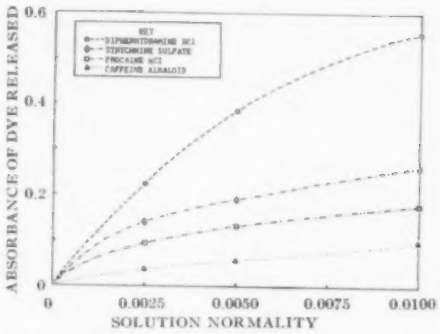

Fig. 4.—Absorbance of dye released from gentian violet bentonite-B by different concentrations of various drug solutions.

the procedure was somewhat modified. Twenty-nine milliliters of acid were added to 200 mg. of GVB-B. The mixture was handled as before, but 0.5 Gm. of sodium acetate was added to 25 ml. of the filtrate to buffer the solution so that the dye color would be constant. The absorbances of these buffered solutions were then determined as before. The absorbances of the dye released by various concentrations of the acids tested are shown in Fig. 5. Large organic cations are held more strongly by a clay than are hydrogen ions (18), in fact Gieseking (1) was unable to replace large organic cations with hydrogen ions. Figure 5 shows that hydrogen ions did replace a large organic cation and that the exchange was related to the strength of the solution used. Since it is the hydrogen ions which do the exchanging, the extent of the exchange may give some indication of the dissociation of the acid. An attempt was made to determine the dissociation constants of mono-, di-, and tri-chloroacetic acids by this method. No correlation was observed, however, with these acids. Since the dissociation equilibrium and the exchange equilibrium are both involved, as the exchange proceeds it will offset the dissociation equilibrium. The anion, which will affect the solubility of the dye, could also interfere with the exchange. Cohn (19) reports that adsorption is not merely governed by the pK value of a compound, but also by less predictable factors such

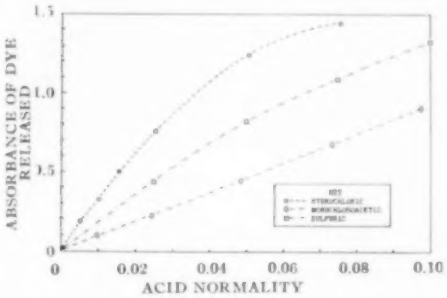

Fig. 5.—Absorbance of dye released from gentian violet bentonite-B by different concentrations of various acids.

as side chains or other steric factors. Kunin and Myers (20) report that dissociation constants can be determined by ion exchange in some cases.

Nature of the Exchange Cations.—Schachtschabel (21) demonstrated that the effects of concentration depend upon the kind of cation that is being displaced and also upon the nature of the replacing ion. Kressman (22) found that the affinities of large organic cations, in contrast to simple metal ions, increased with an increase in ionic size.

The effect of ionic size was tested on a series of straight chain aliphatic amine hydrochlorides. *n*-Propyl, *n*-hexyl, *n*-octyl, and *n*-decyl amine hydrochlorides were prepared by passing HCl gas through a solution of the amine in dry ether. The salts were recrystallized from alcohol and their melting points checked with literature values (23, 24). The salts were dried over phosphorus pentoxide and aqueous solutions of various concentrations were then prepared. The exchange of each solution with GVB-B was then determined using the general method. A plot of the absorbance of the dye released (CSSP) by 0.010 *N* solutions of each salt is shown in Fig. 6. The plot shows that in this series the replacing power of the organic cation increased with the molecular weight of the cation. As can be seen from Fig. 4, when the molecular structure becomes complex, the replacing power does not necessarily follow the molecular weight. Kressman (22) reports that the exchange affinities of some quaternary ammonium compounds increase with the number of atoms in contact with the surface of the exchanger, but that a critical size may be reached where the exchanging ion is larger than the pore size of the exchanger, and where only a limited capacity is shown.

The ability of a random sampling of salts to replace dye from GVB-B showed in general that inorganic cations (sodium, potassium, ammonium, etc.) had low replacing powers while hydrogen and organic cations had high replacing powers.

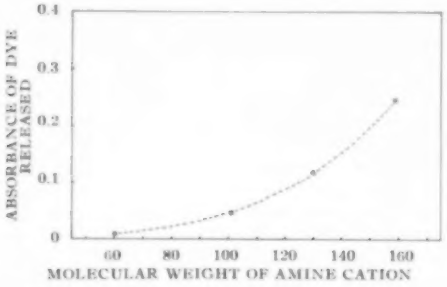

Fig. 6.—Absorbance of dye released from gentian violet bentonite-B by 0.01 *N* solutions of various straight chain aliphatic amine hydrochlorides.

Effect of pH.—Since the hydrogen ion has been shown to be very active in its replacing power, it is possible that pH may affect an organic exchange.

To 400 mg. of GVB-B in a 100-ml. beaker were added 25 ml. of 0.00733 *N* procaine hydrochloride solution. The mixture was then titrated with dilute hydrochloric acid solution until adjusted to pH 1.0. The mixture was then quantitatively transferred to a 50-ml. volumetric flask and brought to volume with distilled water adjusted to the same pH with hydrochloric acid. The same procedure was repeated adjusting the samples to pH values of 2.0, 4.0, 5.0, and 6.0, using the smallest possible amounts of hydrochloric acid or ammonium hydroxide. Ammonium hydroxide was used rather than sodium hydroxide to prevent and minimize possible incompatibility with the gentian violet. The same procedure was repeated with unadjusted solutions, and all of the mixtures were shaken and kept at 25° for one hour and then filtered. Due to possible pH changes, resulting from buffer action or vaporization, the pH of each filtrate was determined. This pH was taken as the pH of the exchange since this was the value at which equilibrium was established. One-half gram of dry sodium acetate was added to 25 ml. of each filtrate to serve as a buffer. The absorbance of these solutions (CSSP) were plotted against the equilibrium pH in Fig. 7.

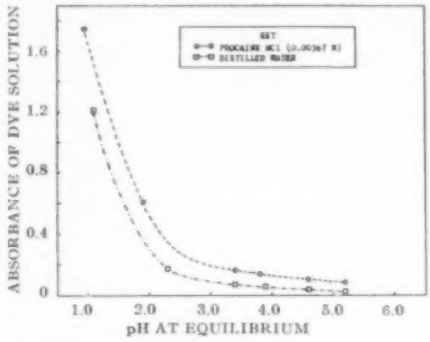

Fig. 7.—Effect of pH on the release of dye from gentian violet bentonite-B by procaine hydrochloride solution.

The distilled water curve shows that more dye was released as the pH was decreased. This corresponds to the statement of Kunin and Myers (20) which reports that adsorption increases with an increase in pH. This curve also follows the pattern of an exchange by hydrogen ions for the dye cations.

The procaine hydrochloride curve follows a similar pattern to that of the distilled water curve. The two curves appear almost parallel, but the difference is accentuated by subtracting the distilled water curve from the procaine hydrochloride curve. The resultant curve (Fig. 8) shows that more dye was released by the procaine hydrochloride solution as the pH decreased.

Reproducibility.—The ability to duplicate the results of a typical exchange is illustrated by a system of GVB-B, a solution of procaine hydrochloride, and for a water blank. A 500-mg. sample of GVB-B was shaken with 50 ml. of each solution. The mixtures were kept at 25 ± 0.5° for one hour and then filtered. The absorbance of each filtrate (CSSP) was determined and found to be 0.078 for distilled water and 0.188 for a 0.00367 *N* procaine hydrochloride solution. The standard deviation was found to be ±0.001 in each case for a series of ten determinations for each solution.

Exchange in Nonaqueous Solvents.—Bodamer and Kunin (25) performed exchange reactions in such

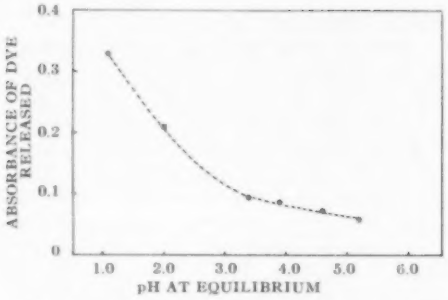

Fig. 8.—Absorbance of dye released from gentian violet bentonite-B by procaine hydrochloride solution at various pH values.

solvents as ethanol, acetone, benzene, petroleum ether, and mineral oil. Therefore, it was decided to study an organic exchange in a solvent such as ethanol. Alcohol removed such a large amount of dye from GVB-B that it was difficult to observe an exchange when alcohol was used as a solvent. To eliminate this difficulty, a new bentonite-B dye complex was prepared. One hundred grams of bentonite-B were mixed with 3 Gm. of powdered gentian violet and 900 ml. of 95% alcohol. The mixture was then treated as in the preparation of GVB-B, except that alcohol was used in place of water at all times. The resulting powder, designated gentian violet bentonite-B (alcohol) or GVB-B-A, was green in color as contrasted to the purple-black of GVB-B, and contained much less gentian violet.

Using this GVB-B-A and different concentrations (0.0025, 0.0050, and 0.0100 *N*) of various drugs (procaine hydrochloride, diphenhydramine hydrochloride, and atropine alkaloid), exchange reactions were measured using the general method. The absorbance of the dye released by each solution (CSSP) was plotted against the solution concentration (Fig. 9). Procaine had a greater replacing power than diphenhydramine in alcohol while the reverse was true when the solvent was water. The solubility of procaine hydrochloride is much less in alcohol than in water, while that of diphenhydramine is only slightly altered. It is felt that these differences in solubility as well as differences in dissociation were responsible for the differences observed in the exchanges with various solvents.

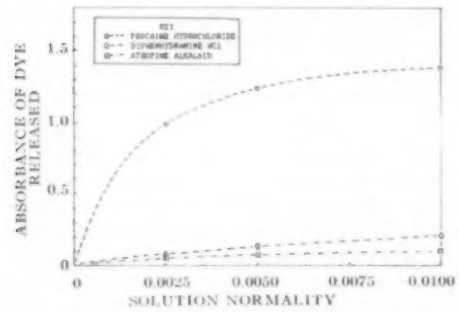

Fig. 9.—Absorbance of dye released from gentian violet bentonite-B (alcohol) by alcoholic solutions of various drugs.

Assay by Desorption.—Since alcohol was found capable of desorbing dye from GVB-B, it seemed possible that there might be some relationship between the amount of dye desorbed and the concentration of the alcohol present. Alcohol solutions of various concentrations were prepared, and their ability to release dye from GVB-B was determined using the standard exchange method. The same procedure was repeated with aqueous solutions of methyl alcohol and of glycerin. The absorbance of the dye released in each case (CSSP) is shown in Fig. 10. The graph shows that the amount of dye released from GVB-B varied with the concentration of organic solvent present and could serve as a means of assay.

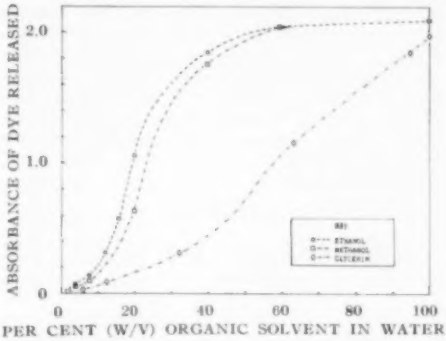

Fig. 10.—Absorbance of dye released from gentian violet bentonite-B by various concentrations of organic solvents in water.

Comparison of Some Exchange Materials.—It is possible that other exchange materials might be more sensitive than bentonite-B for assay purposes. Since Myers (26) pointed out several advantages of synthetic resins, Amberlite IRC-50[2] was also tested. This resin has a rated exchange capacity of 1,000 milliequivalents per 100 Gm. If the extent of an exchange is a direct function of the exchange capacity of the exchanger, this resin should be almost 20 times more efficient than bentonite-B or C.

The resin varied from 16 to 50 mesh and took up very little gentian violet from solution. The Amberlite was ground in a ball mill and passed through a 100-mesh sieve in order to have its particles near the size of the bentonites previously tested. The exchange capacity of this powdered Amberlite was determined in the manner as that used for the clays and found to be 9.48 milliequivalents per 100 Gm. This value is considerably below the rated capacity of the unground resin. As reported by Nachod and Wood (27), the availability of the exchange position is important. This was further emphasized by Kressman (22), who found that when the exchanging ion is larger than the pore size, only a limited capacity is shown. This is quite possible here since methylene blue is a large molecule and the rated exchange capacity of Amberlite is determined using small ions such as hydrogen. The experimental exchange capacity just determined is believed to be more suitable for work with large cations.

[2] A weakly acidic carboxylic exchange resin, The Rohm and Haas Co.

An acidified bentonite was prepared in the same manner as the acid activated bentonite only, after acidifying, the material was simply dried and powdered. This acidified bentonite, which contained all of the exchangeable cations of bentonite plus the hydrogen ions introduced by the acid, was found to have an exchange capacity of 27.6 milliequivalents per 100 Gm.

Gentian violet complexes were made from bentonite-C, acidified bentonite, and powdered Amberlite using the procedure outlined for GVB-B. The dye complexes were washed with distilled water until the washings contained only a faint trace of dye. Exchange reactions were performed with each dye complex and a 0.00367 *N* solution of procaine hydrochloride using the general exchange method. The absorbance of the dye released (CSSP) was found to be 0.083, 0.095, 0.117, and 0.174 for gentian violet Amberlite IRC-50 (100-mesh), gentian violet acidified bentonite, GVB-B, and GVB-C, respectively. These values show some correlation between the amount of dye released and the exchange capacity of the exchanger for organic cations.

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Compression of Crystalline Substances*

JONAH JAFFE† and NOEL E. FOSS

An investigation of the role played by various properties and characteristics of crystals and the ability of these crystals to form tablets when subjected to direct compression was made. Crystal system, binary compounds, water of crystallization, boiling point, carbonate radical, sulfate radical, and substances forming structurally neutral layer lattices, were found to influence tablet formation.

VARIOUS SUBSTANCES such as ammonium chloride, potassium bromide, potassium iodide, potassium chlorate, methenamine, and chloral hydrate, have been known to form tablets upon compression without the addition of foreign ingredients (1).

Very little work has been reported concerning the crystal nature of a substance and its effect on tablet formation, or hardness values of tablets (as tested on the Strong-Cobb Hardness Tester), and on the size of the tablets produced upon direct compression.

In 1951, Kregiel and Foss (2) investigated the role of particle size and crystal system in the formation of tablets. They reported that "the size of particles and bulk density of a powder have a definite influence upon the weight, thickness, density, and disintegration time of compressed tablets," and that "substances belonging to the cubic crystal system presented no difficulty for direct compression."

An attempt to clarify the role played by various properties and characteristics of crystals and the ability of these crystals to form tablets when subjected to direct compression was then made.

An investigation of over 1,500 substances showed that crystal forms are related to molecular structure as follows (3): Cubic and hexagonal crystals are more frequently found in the two and three atom molecules than in the complex molecules or organic molecules; complex molecules with more than five atoms, and organic molecules, tend to form monoclinic crystals; and few substances crystallize in the triclinic system.

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† Abstracted from a thesis submitted by Jonah Jaffe to the Graduate Faculty of the University of Maryland School of Pharmacy, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

This relationship suggested that physical properties may be associated with crystal form. The physical property under investigation is mainly that of tablet formation upon direct compression, and how this property varies under different conditions, such as crystal habit, degree of water of crystallization, chemical relationships, nature of bondings, boiling points, hardness values, and density.

These studies are not prepared to show the feed properties of the various substances tested. To determine the degree of tablet formation, the Strong-Cobb Hardness Tester, designed to show the "hardness" (or the degree of resistance to crushing) of the tablet produced by a Stokes Eureka A-3 single punch tablet machine, manually operated, was employed. Flat head $^{7}/_{16}$ inch punches were used in all cases. Sticking, picking, chipping, capping, etc., were not factors in determining tablet formation in these experiments. The formation of tablets upon direct compression, using the single punch tablet machine, is no indication that such tablets can be formed utilizing a power single punch tablet press or rotary tablet press.

EXPERIMENTAL AND DISCUSSION

Some early workers assumed that homogeneous substances in fine powder form were unsuitable for tablet making (4). Aside from the problem of granular flow (in pharmaceutical sense), the size of the particle has no bearing on its ability to form tablets (5). Consequently, crystals of various sizes, from 20 mesh to fine powders, were subjected to direct compression. The die fill was maintained at constant volume, whereas the pressure was increased for various substances, as needed, to obtain optimum hardness of tablets. Substances were declared unsuitable if a tablet of sufficient hardness (having values of 6 Kg. or more as measured by the Strong-Cobb Hardness Tester) could not be attained upon the exertion of pressure, short of that pressure which would cause jamming of the tablet machine. The tabulation in Table I shows whether the substances formed tablets of sufficient hardness, or of too soft a consistency.

An analysis of Table I seems to show that substances in the cubic system do lend to the formation of tablets upon direct compression, as observed by Kregiel and Foss (2). No such relationship appears in the other crystal systems.

Binary compounds are found predominantly in the cubic and in the hexagonal systems. These substances are characterized by a higher symmetry than ternary and more complex compounds which are found in the rhombic, monoclinic, and triclinic systems.

A relationship thus appears to exist between binary or symmetrical compounds and the property of tablet formation.

Many substances that were subjected to direct compression, have molecules of water of crystallization in their respective formulas.

These molecules of water are bound differently in various crystals. In many cases the water is bound to the cation in an aquo-complex, or as chains traversing the crystal (6). Water of crystallization may be required in a definite position in the crystal lattice, to maintain the stability of the latter (7). Removal of this water of crystallization would then disrupt the crystal structure of the substance. Molecules of water may also occupy holes in the lattice and not be a factor in its stabilization. The role of water of crystallization, in substances subjected to direct compression, was then investigated.

In each case, substances which formed tablets upon compression were unable to show the same property of tablet formation after the removal of the water of crystallization. This indicates that water of crystallization, in some cases, has the same effect as a "built-in" binding agent, holding the various layers of a crystal together. Huckel relates, for example, that water molecules hold together the layers constructed of calcium ions and of tetrahedral sulfate ions in gypsum (8). Those substances which did not form tablets initially, showed no change in tablet formation upon removal of the water of crystallization as shown in Table II.

A definite relationship exists between the pressure applied during compression and the hardness of the tablet formed. Higuchi, *et al.* (9), have recently shown that the "hardness varies directly with the

TABLE I.—DIRECT COMPRESSION OF SUBSTANCES BELONGING TO VARIOUS CRYSTAL SYSTEMS

Substances Which Formed Tablets of Sufficient Hardness			
Acetylsalicylic acid	2[a]	Potassium bromide	1
Aluminum sulfate	2	Potassium chlorate	2
Ammonium alum	1	Potassium chloride	1
Ammonium bromide	1	Potassium iodide	1
Ammonium carbonate	1	Potassium permanganate	3
Ammonium chloride	1	Potassium persulfate	4
Ammonium iodide	1	Potassium tartrate	3
Anthraquinone	3	Salicylic acid	2
Benzoic acid	2	Silver iodide	5
Boric acid (granule)	4	Sodium acetate	2
Calcium oxide	1	Sodium bromide	1
Chloral hydrate	2	Sodium chloride	1
Ferrous sulfate	2	Sodium iodide	1
Iodoform	5	Sodium phosphate	2
Lithium bromide	1	Styphnic acid	5
Magnesium oxide	1	Sulfur	3
Magnesium sulfate	3	Terpin hydrate	3
Mercuric iodide	6	Thiourea	3
Methenamine	1	Urea	6
Naphthalene	2	Zinc Sulfate	3
Substances Which Did Not Form Satisfactory Tablets			
Acetanilid	3	Citric acid	3
Acetophenetidin	2	Ephedrine sulfate	2
Adipic acid	2	Ferric oxide	5
Aminoacetic acid	2	Hydroquinone	5
Ascorbic acid	2	Lactose	2
Barium carbonate	5	Manganese sulfate	2
Barium sulfate	3	Sodium bicarbonate	2
Boric acid (flake)	4	Sodium biphosphate	3
Calcium carbonate	5	Strontium carbonate	3

[a] 1—Cubic system, 2—monoclinic system, 3—rhombic system, 4—triclinic system, 5—hexagonal system, and 6—tetragonal system.

TABLE II.—COMPRESSION OF SUBSTANCES WITH AND WITHOUT WATER OF CRYSTALLIZATION

Substance	Formula	Tablet		Tablet (Water Removed)	
		Good	Poor	Good	Nil
Ammonium alum	$NH_4Al(SO_4)_2.12\ H_2O$	x	..	..	x
Copper sulfate	$CuSO_4.5\ H_2O$	..	x	..	x
Ferrous sulfate	$FeSO_4.7\ H_2O$	x	..	..	x
Magnesium sulfate	$MgSO_4.7\ H_2O$	x	..	..	x
Nickel sulfate	$NiSO_4.7\ H_2O$	..	x	..	x
Potassium alum	$KAl(SO_4)_2.12\ H_2O$	..	x	..	x
Sodium acetate	$C_2H_3NaO_2.3\ H_2O$	x	..	..	x
Sodium carbonate	$Na_2CO_3.H_2O$	..	x	..	x
Sodium phosphate	$Na_2HPO_4.7\ H_2O$	x	..	..	x
Zinc sulfate	$ZnSO_4.7\ H_2O$	x	..	..	x

log of compressionable force, leveling off at high forces."

In the following experiment, various substances were subjected to direct compression (maintaining a constant pressure setting throughout), and the hardness values of the tablets formed were compared. For comparative purposes, approximately equivalent volumes of substances were used, as calculated from their respective densities. All substances were calculated to produce a tablet of 0.335 cc.

Table III lists the various substances, their densities, the calculated volume, the weight of substance subjected to compression, the actual volume of the tablet obtained, and the hardness value in Kg., as determined on the Strong-Cobb Hardness Tester.

TABLE III.—TABLET HARDNESS VALUES AND VOLUME VALUES OF COMPRESSED SUBSTANCES

Substance	Density, Gm./cc.	Weight, Gm.	Actual Volume,[a] Av.	Kg. Strong-Cobb
Aluminum sulfate	1.69	0.566	0.355	30.0
Ammonium bromide	2.429	0.814	0.342	9.0
Ammonium chloride	1.527	0.511	0.350	10.7
Benzoic acid	1.265	0.424	0.336	9.5
Boric acid	1.435	0.481	0.330	5.7
Chloral hydrate	1.908	0.639	0.357	7.8
Copper sulfate	2.28	0.764	0.368	3.7
Iodoform	4.008	1.343	0.348	22.0
Magnesium sulfate	1.63	0.546	0.349	14.0
Naphthalene	1.145	0.384	0.332	9.0
Potassium bromide	2.750	0.921	0.347	19.1
Potassium chloride	1.984	0.665	0.341	24.0
Potassium chlorate	2.32	0.777	0.357	12.0
Potassium iodide	3.13	1.408	0.348	15.2
Potassium permanganate	2.703	0.905	0.354	5.5
Potassium sodium tartrate	1.79	0.600	0.374	23.0
Salicylic acid	1.443	0.483	0.348	4.0
Sodium acetate	1.45	0.486	0.358	12.0
Sodium bromide	3.203	1.073	0.350	16.0
Sodium citrate	1.857	0.622	0.387	3.5
Sulfur	2.07	0.693	0.365	30.0
Thiourea	1.405	0.470	0.343	9.0
Urea	1.335	0.447	0.348	6.5
Zinc sulfate	0.97	0.660	0.349	13.7

[a] Calculated volume is 0.335 cc.

Inspection of Tables III and IV show that substances possess a characteristic "pressure to hardness" ratio. For example, compression of potassium bromide produces a hard tablet, whereas compression of an equivalent volume of sodium citrate (maintaining the same pressure setting) results in a soft tablet. It is also seen that for the same volume and pressure, sodium citrate yields a high volume tablet (0.387 cc.) while powdered aluminum sulfate yields the same volume as calculated, namely 0.335 cc.

TABLE IV.—RELATIVE ORDER OF TABLET HARDNESS VALUES OF SEVERAL SUBSTANCES

Substance	Crystal System	Hardness, Kg.
Sulfur	Rhombic	30.0 plus
Aluminum sulfate	Monoclinic	30.0
Potassium chloride	Cubic	24.0
Potassium sodium tartrate	Rhombic	23.0
Iodoform	Hexagonal	22.0
Potassium bromide	Cubic	19.1
Sodium bromide	Cubic	16.0
Magnesium sulfate	Rhombic	14.0
Zinc sulfate	Rhombic	13.7
Mercuric chloride	Tetragonal	13.5
Sodium acetate	Monoclinic	12.0
Potassium chlorate	Monoclinic	12.0
Ammonium chloride	Cubic	10.7
Benzoic acid	Monoclinic	9.5
Ammonium bromide	Cubic	9.0
Naphthalene	Monoclinic	9.0
Thiourea	Rhombic	9.0
Chloral hydrate	Monoclinic	7.8
Urea	Tetragonal	6.5
Boric acid	Triclinic	5.7
Potassium permanganate	Rhombic	5.5
Salicylic acid	Monoclinic	4.0
Copper sulfate	Triclinic	3.7
Sodium citrate	Monoclinic	3.5

Density does not seem to play any role in hardness values of the substances compressed. Mercuric iodide has a density of 6.283 Gm./cc. and a tablet hardness value of 13.5 Kg. Iodoform, with a lower density of 4.008 Gm./cc., has a higher tablet hardness value of 22 Kg. Potassium chlorate, the lowest of the above mentioned densities (2.32 Gm./cc.) also has the lowest tablet hardness value of 12 Kg. This seems to preclude any relationship between density and tablet hardness values.

The ability of a substance to be inherently cohesive appears to be the main factor determining whether or not a hard tablet can be formed when the substance is compressed under moderate pressure. The degree of cohesive tendency in molecules is related to the volatility of the substance. The more volatile the substance, the lower the cohesive tendency. Richards, *et al.*, indicate that a good guide to the cohesive affinity of a substance is its boiling point (10). Substances with higher cohesive affinity have higher boiling points in order to disrupt the ionic and van der Waal's forces holding the mole-

cules together. Several substances, with their respective boiling points, are tabulated in Table V.

It is seen that a relationship appears to exist between the Strong-Cobb hardness value of the tablet obtained upon compression of a crystalline compound and its boiling point. As the boiling point value goes up, indicating a higher cohesive affinity, the tablet hardness likewise increases.

Those substances containing the sulfate radical (such as barium, calcium, copper, lead, manganese, nickel, and potassium sulfates) do not tend to form tablets upon direct compression. The nature of the bonding may play an important role here, as the sulfate radical has a combination of normal covalent and coordinate links (11).

It was previously indicated that compounds showing high symmetry, as exhibited by the binary compounds, show good tablet formation.

If carbonates are visualized as low symmetry distorted structures (12) they may show a poor tendency to form tablets upon direct compression (in contrast to the high symmetry binary compounds). The carbonates of calcium, lead, nickel, potassium, silver, and sodium did not form tablets.

Talc, a layer structure which has no interfacial binding (13), does not form a tablet. Kaolin, a similar layer structure, does not form a tablet either. The loose superposition of these neutral layers form soft substances which are easily cleaved (14). Mercuric iodide, which forms a layer lattice has "bridge" atoms holding the various layers together (15). This indicated that a tablet of mercuric iodide might be formed. This was shown to be the case.

TABLE V.—RELATIONSHIP OF TABLET HARDNESS AND BOILING POINTS OF SEVERAL SUBSTANCES

Substance	Hardness, Kg.	Boiling Point, °C.
Potassium chloride	24.0	1,500
Potassium bromide	19.1	1,380
Sodium bromide	16.0	1,390
Potassium iodide	15.2	1,420
Mercuric iodide	13.5	354
Benzoic acid	9.5	249
Naphthalene	9.0	218
Chloral hydrate	7.8	96.3

SUMMARY

1. A relationship appears to exist between binary compounds and the property of tablet formation. Only substances of the cubic system show a general pattern of tablet formation upon direct compression.

2. Water of crystallization acts as a "built-in" binding agent in various substances. Removal of this water prevents tablet formation (upon direct compression).

3. Density does not seem to play any role in the tablet hardness values of the substances compressed.

4. A relationship appears to exist between the cohesiveness of a substance as manifested by its boiling point and the tablet hardness value the substance achieves upon compression.

5. Those substances containing the sulfate radical do not tend to form tablets upon direct compression.

6. Those substances containing the carbonate radical show a poor tendency to form tablets upon direct compression.

7. An indication exists that substances forming structurally neutral layer lattices do not lend to tablet formation.

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A Study of the Gravimetric Silicotungstate Method of Assay for Thiamin*

By ENOS E. VANNATTA† and LOYD E. HARRIS

The factors affecting the method of the 1953 British Pharmacopoeia for the assay of thiamin have been investigated. An improved procedure is presented, and its application is demonstrated. The advantages of the proposed method, with respect to the fluorometric and other methods with which it has been compared, are: greater accuracy, increased specificity, less time-consuming, inexpensive apparatus and reagents, less tedious operations, and direct interpretation of data which does not involve the use of a reference standard.

NUMEROUS analytical methods, including biological, microbiological, fermentative, physical, and chemical, have been employed for the assay of thiamin (1). The most important of these in pharmacy is the chemical group, which includes colorimetric, fluorometric, gravimetric, and volumetric procedures. A colorimetric method, based upon the coupling of thiamin with diazotized *p*-aminoacetophenone, was official in the U. S. Pharmacopeia XII. Subsequent revisions have adopted a fluorometric method which is based upon the fluorescence of thiochrome, an oxidation product of thiamin. Although this method has been thoroughly investigated and many modifications have been proposed, it is still subject to criticism (2–4). The procedure is tedious and time-consuming, and it requires special equipment. Slight variations in experimental conditions make it necessary that a standard be run simultaneously with each sample, and even then the results lack good precision.

Since present-day pharmaceutical products are compounded almost exclusively with pure, synthetic thiamin and their potency is many times that of the naturally occurring substances for which the fluorometric method was originally developed, it was considered advisable to devise a method of greater accuracy and simplicity than that provided by the official method.

Bessot (5), in 1939, suggested a gravimetric method using silicotungstic acid as the precipitant. Adamson and Handisyde (3), in 1948, in Great Britain, after having investigated certain of the experimental factors and the effect of other vitamins, synthetic intermediates, and tablet excipients, reported a modification of Bessot's procedure to be a satisfactory method for the determination of thiamin in ampul and tablet preparations. Although apparently there has been no further publication pertaining to the method, that which Adamson and Handisyde had proposed was adopted, without alteration, by the 1953 British Pharmacopoeia for the assay of aneurine hydrochloride and for ampuls and tablets thereof.

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EXPERIMENTAL

Factors Affecting the British Pharmacopoeia Method

The B. P. procedure for aneurine hydrochloride is as follows: "Dissolve about 50 mg., accurately weighed, in 50 ml. of water, add 2 ml. of hydrochloric acid, and heat to boiling. To the boiling solution, rapidly add drop by drop 4 ml. of solution of silicotungstic acid (10 per cent), boil for two minutes, filter through a sintered glass crucible and wash with a boiling mixture of 1 volume of hydrochloric acid and 19 volumes of water, and then with 10 ml. of water and two quantities, each of 5 ml., of acetone. Dry the residue to constant weight at 105°. Each g. of residue is equivalent to 0.1936 g. of $C_{12}H_{17}ON_4SCl$-HCl."

Because of the very limited available information regarding this method, the factors affecting it were investigated in detail.

Reagents.—Thiamin hydrochloride, ampul-grade, dried at 105° for three hours; 10% silicotungstic acid solution; dilute hydrochloric acid (1:24); acetone, reagent grade.

Amount of Silicotungstic Acid Required.—Thiamin and silicotungstic acid combine in a molar ratio of 2 to 1, respectively. Thus the theoretical amount of the precipitant required is 4.95 mg. per mg. of thiamin hydrochloride.

Several aliquots of a thiamin solution were assayed by the B. P. method using varied amounts of the silicotungstic acid solution. The results, which are shown in Table I, indicate that the 60% excess as specified in the B. P. procedure is adequate. However, since the results were slightly higher for the 50-mg. than for the 25-mg. aliquots at the same per cent excess of precipitant, a volume excess of 1.5 ml. is recommended in the proposed method.

Effect of Boiling Time.—The boiling period following the addition of the silicotungstic acid is not critical. The results obtained for 50-mg. samples at boiling periods from 1 to 5 minutes did not differ significantly. A disadvantage of prolonged boiling is that a portion of the precipitate

TABLE I.—AMOUNT OF SILICOTUNGSTIC ACID REQUIRED

Thiamin Taken, mg.	Silicotungstic Acid Solution, ml.	Excess[a] ml.	Excess[a] %	Thiamin Recovered,[b] %
25.4	1.5	0.25	20	98.1
25.4	1.5	0.25	20	97.6
50.8	3.0	0.50	20	98.7
25.4	2.0	0.75	60	98.1
25.4	2.0	0.75	60	98.4
50.8	4.0	1.50	60	99.0
25.4	2.5	1.25	100	98.6
25.4	2.5	1.25	100	98.9
25.4	3.0	1.75	140	98.9
25.4	3.0	1.75	140	98.7

[a] Excess over approximate theoretical amount.
[b] Precipitate dried at 105°.

adheres to the upper walls of the beaker and makes its transfer more difficult. Insufficient boiling may yield an extremely fine precipitate which is difficult to filter.

Effect of Wash Solvents.—The 50 ml. of water and the 2 ml. of hydrochloric acid used in the B. P. procedure for dissolving the sample and the dilute hydrochloric acid (1:19) used for transferring and washing the precipitate were replaced with dilute hydrochloric acid (1:24) to simplify the preparation of reagents.

Preliminary tests involving the rewashing, drying, and weighing of previously dried and weighed precipitates indicated an error due to the solubility of the precipitate in the boiling acid wash. Further tests using varied quantities of the wash solvent for the assay of similar samples verified this observation and also indicated that the error could be reduced by the addition of a small amount of silicotungstic acid to the boiling acid wash. When it was observed that the filtrate from the water wash was occasionally turbid, indicating a physical attrition of the precipitate, the water was replaced with the dilute hydrochloric acid (1:24). The solubility in acetone was negligible.

The loss of thiamin in the original sample filtrate, the 50-ml. boiling acid wash, with and without the addition of silicotungstic acid, and the three 5-ml. water or acid washes was determined by the fluorometric method. The results are given in Table II and indicate the advantages of the proposed changes in the B. P. washing procedure.

Effect of Drying Temperature.—Bessot proposed the following molecular formula for the thiamin silicotungstate precipitate: $12WO_3 \cdot SiO_2 \cdot (OH)_2 \cdot (C_{12}H_{17}N_4OS)_2 \cdot 4H_2O$. The corresponding molecular weight, using the atomic weights of the 1954 International Committee of Atomic Weights, is 3479.94. Thus the factor for conversion of a known weight of the precipitate to the equivalent weight of thiamin hydrochloride is 0.1938 (the B. P. factor is 0.1936). The corresponding factor for conversion to thiamin mononitrate is 0.1882. Since these factors are based upon the presence of four molecules of water in the molecular formula of the precipitate, the effect of the temperature at which the precipitate is dried was carefully studied.

A quantity of thiamin silicotungstate was prepared and allowed to dry at room temperature for twenty-four hours. Two samples of this air-dried

TABLE II.—SOLUBILITY OF PRECIPITATE IN ORIGINAL FILTRATE AND AQUEOUS WASH SOLVENTS

Solution Analyzed	Thiamin Loss, mg.	Precipitate Loss, mg.	Error for a 50-mg. Sample, %
Original sample filtrate,	0.058	0.300	0.12
	0.055	0.280	0.11
	0.057	0.290	0.11
Boiling acid wash, 50 ml.	0.137	0.710	0.27
	0.102	0.530	0.20
	0.117	0.600	0.23
Boiling acid wash, 50 ml., with 1 ml. of silicotungstic acid solution added	0.010	0.052	0.02
	0.011	0.057	0.02
	0.010	0.053	0.02
Three 5-ml. washes of water	0.036	0.190	0.07
	0.171[a]	0.880	0.34
	0.023	0.120	0.05
Three 5-ml. washes of dilute acid	0.000	0.000	0.00
	0.003	0.020	0.01

[a] Turbid.

material, each in a tared weighing bottle, were dried to constant weight at several different temperatures. The loss in weight at each temperature is shown in Fig. 1. It was found that the samples rapidly returned to their initial weights on exposure to atmospheric moisture. The increases in weight for the two samples, each having been dried at a different temperature, are shown in Fig. 2. The loss and recovery of this small amount of water was reversible and reproducible over the entire temperature range tested. The increase in weight on exposure to air is important since assay precipitates in filtering crucibles are exposed during the time that they are being weighed. This is between 0.2 and 0.4% for precipitates dried at 80° and weighed within two minutes after removal from a desiccator. Constant weight at 80° is attained in three hours.

The two samples which had been used in the loss

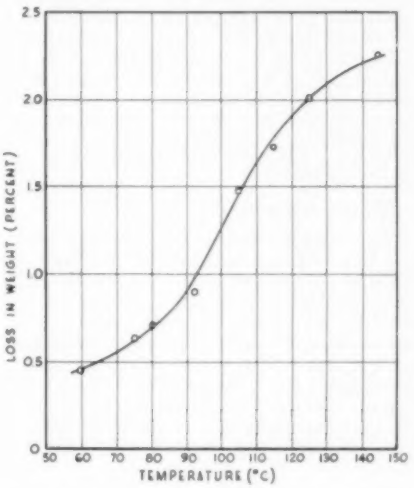

Fig. 1.—Loss in weight of air-dried thiamin silicotungstate at different drying temperatures.

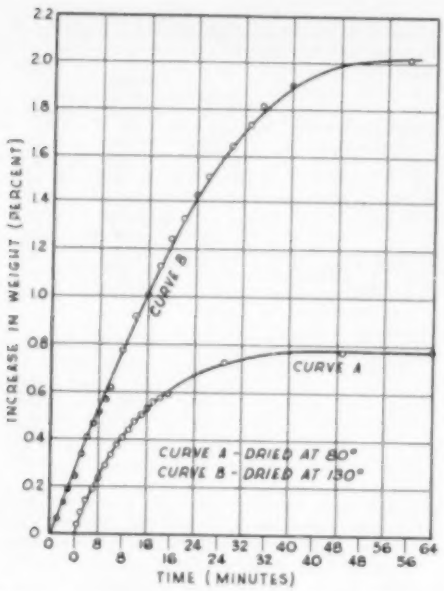

Fig. 2.—Increase in weight of dried thiamin silicotungstate on exposure to air.

on drying experiments were allowed to return to their air-dried weights. Then they were transferred to platinum crucibles, reweighed, and their silicotungstic anhydride content determined by ignition to constant weight at 800°. The theoretical silicotungstic anhydride content of completely anhydrous thiamin silicotungstate is 83.43%. From these data and those shown in Fig. 1, the water content of the precipitate and the corresponding molecular weight at each of the different drying temperatures were calculated. These are listed in Table III. It is apparent that a drying temperature of 105° results in a loss of approximately 60% of the 4 moles of water and thereby introduces an error of 1.25% if the conversion factors, 0.1938 and 0.1882, are used. Drying at 80° removes about 22% of the molecular water and produces an error of about 0.45%. However, this is largely counterbalanced by the fairly uniform increase in weight while weighing.

Interfering Substances

Other vitamins and closely related substances, trace mineral ions, a large group of both organic and inorganic anions, and several nitrogen-containing compounds were investigated for possible interference. As was to be expected, many amine-type compounds were precipitated at room temperature, but not under the conditions of the proposed method and in the concentrations in which they are normally present. Interference from betaine, choline, or niacinamide is most likely since they frequently occur with thiamin in relatively high concentrations. However, 10 mg. per ml. of any of these do not precipitate under the proposed conditions. Alkaloids and antihistamines may interfere, but these are seldom combined with thiamin, and then only in low concentrations. Amphetamine, methamphetamine, and ephedrine were found not to interfere in concentrations up to 2 mg. per ml. None of the other substances tested proved troublesome.

The possibility of interference from synthetic intermediates[1] was investigated. One of these, thiamin bromide hydrobromide, was precipitated quantitatively. Three other intermediates in the final stages of the commercial synthesis of thiamin, representing both the pyrimidine and thiazole portions of the thiamin molecule, were tested. These were 4-methyl-5-(-hydroxyethyl) thiazole, 2-methyl-4-hydroxy-5-methoxy methylpyrimidine, and the 4-amino analog of the latter compound. While each of these was precipitated by silicotungstic acid at room temperature, no precipitate resulted at the boiling temperature when present in concentrations up to 2 mg. per ml. Fifty milligrams of thiamin hydrochloride in the presence of 10 mg. of each of the three compounds assayed 100.6% by the proposed method. Thus the method differentiates between thiamin and traces of these substances as impurities or larger quantities as decomposition products.

Fifty-milligram samples of thiamin hydrochloride were partially decomposed by heating for different periods of time in alkaline media. Then the solutions were acidified and assayed. Although there was some interference or coprecipitation in the samples of greater decomposition, as indicated by the tan or light brown instead of the normal white color of the precipitate, the results in Table IV show a comparative and certainly significant differentiation once again between the intact thiamin and its decomposition products.

Liquid products of thiamin frequently contain alcohol. However, the results obtained for samples precipitated in the presence of as much as 20% of this solvent were no different from those with no alcohol.

The most frequent source of interference to the direct application of the proposed method arises from the presence of such substances as stomach concentrates, liver and bran extracts, brewer's yeast, malt syrups, oils, and substances used in the formulation of certain products, especially of soft gelatin capsules and liquids. The high viscosity of sample solutions, or the decomposition with resulting precipitation, or the coagulation of these ingredients necessitate the preliminary isolation and concentration of the thiamin. This has been accomplished by an ion-exchange technique, using the carboxylic, cation-exchange resin, Amberlite IRC-50, which will be described in a subsequent paper.

Proposed Method of Assay and Its Application

Certain changes in the method of the 1953 British Pharmacopoeia have been found necessary. The proposed procedure is directly applicable to bulk thiamin hydrochloride, thiamin mononitrate, and to those preparations which are not subject to the physical interference referred to in the preceding section.

Procedure.—Dilute ampul and other liquid preparations with dilute hydrochloric acid (1:24),

[1] The intermediates tested were furnished through the courtesy of Merck & Co.

TABLE III.—MOLECULAR WEIGHT OF THIAMIN SILICOTUNGSTATE AT DIFFERENT DRYING TEMPERATURES

Drying Temperature, °C.	Weight of Thiamin Silicotungstate, Gm. Sample 1	Sample 2	Silicotungstic Anhydride Content,[b] % Sample 1	Sample 2	Moles of Water Sample 1	Sample 2	Molecular Weight Sample 1	Sample 2
Air-dried	1.2088[a]	0.9421[a]	81.46	81.53	4.57	4.40	3490.16	3487.10
60	1.2032	0.9378	81.84	81.90	3.67	3.51	3474.00	3471.17
80	1.2002	0.9354	82.04	82.11	3.19	3.02	3465.33	3462.29
105	1.1909	0.9282	82.69	82.75	1.70	1.54	3438.49	3435.64
125	1.1845	0.9232	83.13	83.20	0.67	0.51	3420.00	3417.14
145	1.1815	0.9208	83.34	83.42	0.19	0.02	3411.34	3408.26

[a] These weights were experimentally determined. All other weights were calculated from them, based upon the data in Fig. 1.
[b] When ignited at 800°, the samples gave residues as follows: Sample 1—0.9847 Gm. and Sample 2—0.7681 Gm.

TABLE IV.—EFFECT OF PRODUCTS RESULTING FROM DECOMPOSITION OF THIAMIN IN ALKALINE SOLUTION

Thiamin Taken, mg.	Heating Time, min.	—Thiamin Recovered— mg.	%
50.09	0	50.03	99.9
49.55	0	49.46	99.8
50.09	15	33.55	67.0
49.55	15	31.34	63.2
50.09	30	20.52	41.0
49.55	30	19.63	39.6
50.09	60	12.25	24.5
49.55	60	11.78	23.8

or with water and an equivalent amount of concentrated hydrochloric acid, to a concentration of 0.5 to 1.0 mg. of thiamin per ml. Extract capsule and tablet samples with the dilute acid solvent by conventional procedures. Transfer 50 ml. of the sample solution containing 25 to 50 mg. of thiamin to a 150-ml. beaker. Heat the solution to boiling, and add rapidly, but dropwise, 0.5 ml. of 10% silicotungstic acid solution for each 10 mg. of thiamin plus an additional 1.5 ml. Continue to boil gently while stirring for three minutes. Filter through a tared, 30-ml. sintered-glass crucible of fine porosity, transfer, and wash the precipitate with a boiling mixture of 50 ml. of the dilute hydrochloric acid (1:24) and 1 ml. of the silicotungstic acid solution in approximately 5-ml. portions. Then wash the precipitate with three 5-ml. portions of the dilute acid, being careful to direct the solution around the sides of the crucible and to wet the entire precipitate with each portion. Draw air through the crucible for one to two minutes and finally wash with two 5-ml. portions of acetone. Dry the crucible and contents at 80° for three hours, cool, and weigh. Each Gm. of residue is equivalent to 0.1938 Gm. of $C_{12}H_{17}ClN_4OS \cdot HCl$ or 0.1882 Gm. of $C_{12}H_{17}N_4OS \cdot HNO_3$.

The method was applied to the analysis of commercial bulk thiamin and several diversified products. The results obtained and the other labeled ingredients of the products are given in Table V.

TABLE V.—ASSAY OF THIAMIN PRODUCTS BY PROPOSED GRAVIMETRIC METHOD

Product No.	Thiamin Content Labeled, mg.	Found, %	Type Product and Other Labeled Ingredients
1	...	99.6	Bulk thiamin hydrochloride
		99.7	
		99.9	
2	...	99.3	Bulk thiamin mononitrate
		99.7	
		99.9	
3	5	107.9	Tablet
		108.1	
		108.3	
4	25	109.4	Tablet
		110.0	
		110.5	
5	5	118.9	Tablet; 5 mg. desoxyephedring HCl and 45 mg. thyroid
		119.6	
		120.7	
6	6.66 (per oz.)	99.0	Elixir; 10% alcohol
		99.6	
		100.0	
7	5 (per 5 ml.)	94.2	Elixir; 16 mg. phenobarbital; 15% alcohol
		94.9	
8	10 (per 5 ml.)	104.4	Solution; 25 μg. vitamin B_{12}
		104.8	
		104.8	
9	100 (per ml.)	113.8	Ampul; 100 mg. pyridoxine HCl
		114.1	
		114.7	
10	25 (per ml.)	114.9	Ampul; 2 mg. riboflavin, 100 mg. niacinamide, 75 mg. choline chloride, 15 mg. inositol, 25 mg. methionine, and 5 μg. vitamin B_{12}
		115.2	
		115.4	
11	33 (per ml.)	116.7	Ampul; 1 mg. riboflavin, 50 mg. niacinamide, 0.1 mg. pyridoxine HCl, and 1 mg. calcium pantothenate
		116.8	
		116.9	
12	10 (per ml.)	117.8	Ampul; 5 mg. riboflavin, 200 mg. niacinamide, 2.5 mg. pyridoxine HCl, 2.5 mg. calcium pantothenate, and 15 mg. ferric cacodylate
		119.0	
		119.5	

Comparison with Other Methods

Five of the products assayed by the proposed gravimetric method were also tested by three other chemical methods. These included the official fluorometric method of the U. S. Pharmacopeia XV, a colorimetric method (6) involving a coupling reaction with diazotized *p*-amino-acetophenone, and a volumetric, nonaqueous titration method (4) using perchloric acid as the titrant. The results listed in Table VI show close agreement of the gravimetric method with the others and greater precision, especially in comparison to the fluorometric method.

SUMMARY

The gravimetric method of the 1953 British Pharmacopoeia for the assay of thiamin hydro-

TABLE VI.—COMPARISON OF DIFFERENT METHODS OF ASSAY

Product No.	Labeled Thiamin Content, mg.	Found Thiamin Content: Gravimetric, %	Fluorometric, %	Colorimetric, %	Volumetric,[a] %
1	Bulk	99.6	99.8	100.4	100.0
		99.7	101.0	100.6	99.9
		99.9	102.0	100.8	100.2
4	25 (per tab.)	109.4	106.4	106.0	110.6
		110.0	110.0	108.0	111.8
		110.5	115.2	108.5	112.0
5	5 (per tab.)	118.9	117.2	113.6	...
		119.6	118.4	114.4	...
		120.7	121.6	114.8	...
9	100 (per ml.)	113.8	112.0	115.7	...
		114.1	113.5	116.0	...
		114.7	116.9	116.3	...
12	10 (per ml.)	117.8	118.1	116.8	...
		119.0	118.8	117.3	...
		119.5	120.8	118.0	...

[a] Products 5, 9, and 12 could not be assayed by this method since other basic ingredients were present.

chloride has been investigated and a modification of the procedure is proposed. The method has been improved by the addition of a small quantity of the precipitant, silicotungstic acid, to the boiling acid used for transferring and washing the precipitate and by decreasing the drying temperature from 105 to 80°.

The method has been shown to be free of interference from most of the other vitamins and substances frequently associated with thiamin in pharmaceutical products with the possible exception of betaine, choline, or niacinamide if present in unusually high concentrations. Interference of a physical nature by crude extracts and concentrates or formulating agents necessitates the preliminary separation of the thiamin. This may be accomplished by the use of an ion-exchange technique, the procedure for which will be presented in a separate paper. The method has been shown to differentiate between thiamin and possible impurities or decomposition products.

Representative products have been assayed by the proposed method, and the results have been compared with those obtained by the official fluorometric, a colorimetric, and a volumetric method and found to be in close agreement.

The method offers these significant advantages: greater accuracy, increased specificity, less time-consuming, inexpensive apparatus and reagents, less tedious operations, and direct interpretation of data which does not involve the use of a reference standard.

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The Gravimetric Silicotungstate Method of Assay for Thiamin in the Presence of Interfering Substances*

By ENOS E. VANNATTA† and LOYD E. HARRIS

The adsorption and the subsequent elution of thiamin from columns of a buffered ion-exchange resin, Amberlite IRC-50, have been investigated. The procedure has been applied to the separation of thiamin and its gravimetric determination, when interfering substances are present.

A GRAVIMETRIC METHOD of assay for thiamin has been described (1). Compared to the fluorometric and other chemical methods, it offers several advantages, the most important of these being greater accuracy. However, it is not directly applicable to products containing ingredients such as liver and bran extracts, stomach concentrates, brewer's yeast, malt syrups, oils, and other substances used in their formulation.

Synthetic ion-exchange resins are used in the recovery, separation, and analysis of many substances. Since the manufacturer of Amberlite IRC-50, a weakly acidic, carboxylic, cation-exchange resin, had indicated an efficiency of 92 to 95 per cent in the adsorption of thiamin by the sodium-form of this resin (2), its application to the separation of thiamin from substances which interfere in the gravimetric assay was investigated. Two characteristic and essential properties of Amberlite IRC-50 are: adsorbed ions can be effectively eluted by moderate concentrations and volumes of acid, and it can easily be buffered, thereby affecting its adsorptivity for weakly basic compounds.

* Received September 4, 1958, from The Ohio State University, College of Pharmacy, Columbus 10.
An abstract from a portion of a thesis submitted by Enos E. Vannatta to the Graduate School, The Ohio State University, in partial fulfillment of the requirements for the degree of Master of Science.
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EXPERIMENTAL

Reagents.—Amberlite IRC-50, analytical grade; 0.1 *N* H_2SO_4; 1.0 *N* NaOH; pH 4.5 buffer solution (11.7 Gm. of sodium acetate and 6.9 ml. of glacial acetic acid per 100 ml.); 10% sodium hydroxide; 15% sodium acetate; 5% hydrochloric acid; reagents for gravimetric method of assay.

Preparation of Resin Column.—Transfer about 10 Gm. of the resin, as supplied in the moist, hydrogen-form, to a stoppered flask, add 40 ml. of 10% sodium hydroxide, and shake thirty minutes. Decant the excess alkali solution, and rinse the resin with 2 or 3 portions of water. Transfer 25 ml. of this sodium-form of the resin to a beaker using 50 ml. of 15% sodium acetate solution, and adjust the pH of the mixture, while stirring continuously, to the desired value by the intermittent addition of acetic acid until the pH remains constant for at least thirty minutes. Allow the buffered resin to stand overnight in the acetate solution, and then transfer it to a 50-ml. buret containing a pledget of glass wool as a support. Next backwash the resin with about 150 ml. of water to remove air bubbles and layer the smaller particles at the top of the column. Then allow the resin to settle and wash the column with about 100 ml. of water, after which it is ready for use. The resin for several columns may be converted to the sodium-form and buffered as a single portion by increasing the reagents used proportionately.

Optimum Adsorption Conditions.—The adsorption of thiamin on columns of buffered Amberlite IRC-50 was studied with respect to the pH of the resin and the rate of flow of the thiamin solution. Solutions containing 50 mg. of thiamin hydrochloride in 100 ml. of 0.1 *N* H_2SO_4 were adjusted to a pH of 4.5 by the addition of a mixture of 10 ml. of 1.0 *N* NaOH and 12.3 ml. of the pH 4.5 buffer solution. These solutions were passed through columns of resin, which had been buffered at pH values ranging from 5.0 to 6.5, at rates of flow from 1.5 to 6.0 ml. per minute. The columns were washed with 15 ml. of water at the same rate of flow as the thiamin solution and then with 75 ml. of water at a rate of 5 to 6 ml. per minute. The effluent solutions for each column were combined, and the thiamin content was determined fluorometrically. The loss or leakage of thiamin with respect to the pH at which the resin for different columns was buffered and the rate of flow of the thiamin solution is presented in Fig. 1.

Since it was found desirable also to wash the columns obtained from certain products with mixtures of acetone and water to facilitate the removal of various interfering substances, the effect of such treatment was examined. Two of the columns of resin from the above tests, one of which had been buffered at a pH of 5.4 and the other at 6.0, were washed at a rate of 5–6 ml. per minute, successively, with 25 ml. of acetone-water (1:2), 75 ml. of acetone-water (2:1), 25 ml. of acetone-water (1:2), and 100 ml. of water. The combined thiamin content of these additional washes was only 11.4 μg. for each column.

The presence of alcohol in liquid preparations containing thiamin prompted a study of its effect on adsorption. The leakage which resulted for thiamin solutions of the same composition as previously used, but with 2.5 to 15% alcohol added, was determined for resin buffered at a pH of 5.5 at rates of flow of 2.5 and 4.0 ml. per minute. The results, shown in Fig. 2, indicate an increased loss which, however, is less than 0.2% for solutions containing not more than 8% alcohol at a rate of flow of 2.5 ml. per minute or less.

Elution and Recovery.—It was necessary that a satisfactory procedure for the elution of the thiamin meet two conditions; namely, quantitative elution and a concentration of acid in the eluate approxi-

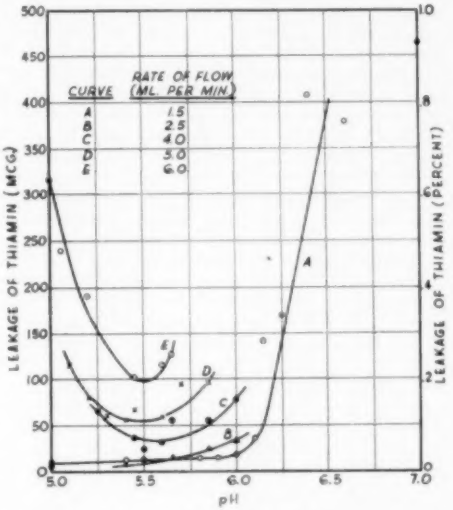

Fig. 1.—Adsorption of thiamin by Amberlite IRC-50 buffered at different pH values and at different rates of flow.

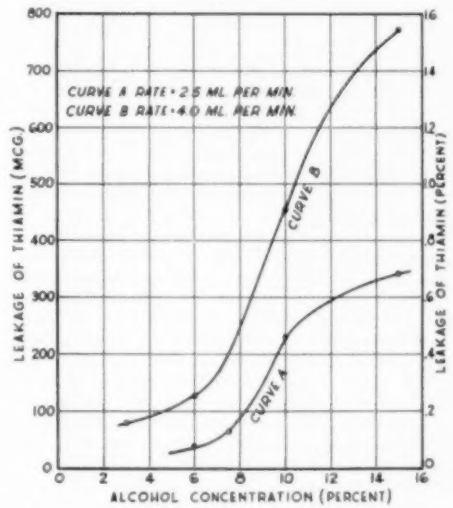
CURVE A RATE • 2.5 ML. PER MIN.
CURVE B RATE • 4.0 ML. PER MIN.
CURVE B
CURVE A
LEAKAGE OF THIAMIN (MCG.)
LEAKAGE OF THIAMIN (PERCENT)
ALCOHOL CONCENTRATION (PERCENT)

Fig. 2.—Effect of alcohol on adsorption of thiamin by Amberlite IRC-50 (buffered at a pH of 5.5).

mately equal to that of the dilute hydrochloric acid used as the solvent in the gravimetric method of assay. It was established by the elution of several of the columns from the study of adsorption conditions that the use of 5% hydrochloric acid at a rate of 1 ml. per minute until the eluate became acidic to methyl orange, followed by 60 ml. of dilute hydrochloric acid (1:24) at the same rate, would fulfill both of these requirements.

Solutions were prepared by dissolving 50 mg. of thiamin in 100 ml. of 0.1 *N* H_2SO_4 and subsequent addition of a mixture of 10 ml. of 1.0 *N* NaOH and 12.3 ml. of pH 4.5 buffer. These solutions were passed through columns of resin buffered at a pH of 5.6 at a rate of flow of 4 ml. per minute. Each column was washed with 15 ml. of water at a rate of 4 ml. per minute and then, successively, at a rate of 5 to 6 ml. per minute, with 50 ml. of water, 15 ml. of acetone-water (1:2), 50 ml. of acetone-water (2:1), 15 ml. of acetone-water (1:2), and 50 ml. of water. The columns were eluted with 5% hydrochloric acid until the eluate became acidic to methyl orange and then with 50 ml. of dilute hydrochloric acid (1:24) at a rate of 1 ml. per minute. The thiamin content of the eluate from each column was determined by the gravimetric method. The elution of the columns was continued with a 10-ml., and then a 50-ml. portion of the dilute acid to yield eluates A and B, respectively, and the thiamin content of each was determined fluorometrically. These results and those for the thiamin recovered in the initial eluates are given in Table I.

TABLE I.—ELUTION AND RECOVERY OF THIAMIN FROM RESIN

Sample	Thiamin Taken, mg.	Thiamin Content Tests for Completion of Elution			Thiamin Recovered,* %
		Eluate	mg.	%	
1	51.04	A	0.030	0.06	99.71
		B	0.019	0.04	
2	50.20	A	0.047	0.09	99.88
		B	0.015	0.03	
3	51.04	A	0.080	0.16	99.55
		B	0.039	0.08	
4	50.20	A	0.021	0.06	99.64
		B	0.019	0.04	

* From initial eluates.

Application of Modified Assay Procedure.—The thiamin, in a representative group of products, was first separated from interfering substances using the resin and then assayed by the gravimetric method. The results are given in Table II.

The procedure for the preparation of sample solutions depended upon the characteristics of each particular product; this was especially true for soft gelatin capsules. Liquid samples were diluted with water and an appropriate volume of the pH 4.5 buffer to yield a thiamin concentration of about 0.5 mg. per ml. and an alcohol content of not more than 5%. The volume of pH 4.5 buffer used should be 10% of the volume of the diluted sample before it is passed through the column. When applicable, the extraction procedures of the U. S. P. XV for related products were followed for tablets and capsules. Soft gelatin capsules for which U. S. P. extraction procedures were not suitable were extracted by vigorous shaking with successive portions of hot 0.1 *N* H_2SO_4, decanting through a layer of Celite filter-aid. Coated tablets containing large amounts of basic substances may require the use of a more concentrated acid than 0.1 *N* H_2SO_4. Samples were selected to yield 15 to 25 mg. of thiamin. The amount of 1.0 *N* NaOH to be mixed with the pH 4.5 buffer in adjusting the pH of the sample was determined by titration of a duplicate sample or sample aliquot to that pH. The mixture of buffer and hydroxide was added to the sample, and the solution[1] was passed through a column of resin, buffered at a pH of 5.6, at a rate of 2 to 3 ml. per minute. The column was then washed, the thiamin eluted with 5% hydrochloric acid and 60 ml. of the dilute acid (1:24), as described previously, and the eluate assayed by the silicotungstate method (1). Three ml. of the silicotungstic acid solution was used for precipitating quantities of 25 mg. or less of thiamin.

TABLE II.—ASSAY OF THIAMIN PRODUCTS BY MODIFIED GRAVIMETRIC PROCEDURE

Type Product	Thiamine Content—Labeled, mg.	Thiamine Content—Found, %
B-complex, hard gelatin capsule	2.5	118.0
		119.0
		119.4
Multiple vitamin, hard gelatin capsule	5	95.3
		95.5
Multiple vitamin, soft gelatin capsule	10	114.9
		115.5
		115.8
Multiple vitamin, soft gelatin capsule with trace minerals	3	106.6
		107.1
		109.2
B-complex tablet with iron	1	111.4
		111.9
		113.1
Multiple vitamin tablet	10	120.0
		120.2
		120.7
B-complex elixir with liver concentrate, iron, and 10% alcohol	18 (per oz.)	119.8
		122.2
B-complex elixir with rice bran concentrate and 7% alcohol	12 (per oz.)	115.6
		116.8
		118.3

SUMMARY

The gravimetric method of assay for thiamin is not directly applicable to products containing crude extracts and concentrates or various formulating agents. However, the method can be used after a preliminary separation of the thiamin from these interfering substances.

Optimum conditions for the adsorption of thiamin on columns of buffered Amberlite IRC-50 have been determined. The subsequent, quantitative elution of the thiamin is described.

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[1] Oils, suspended in solution after filtration, can be removed by swirling the solution with a small volume of toluene.

A Phytochemical Investigation of *Rauwolfia decurva* Hook.*

By CHAND K. ATAL†

From the roots of *R. decurva* Hook., reserpiline, isoreserpiline, reserpine, and sarpagine have been isolated. In addition to these alkaloids, probable presence of rescinnamine, reserpinine, and two unidentified alkaloids have been shown by paper chromatographic evidence. Ajmaline, which forms a major alkaloid in the closely related species *R. densiflora*, is conspicuously absent in the species under study.

IN A PREVIOUS PUBLICATION (1), the pharmacognostic characters of *Rauwolfia decurva* Hook. were described. This species is indigenous to India and is taxonomically very closely related to *R. densiflora*. From the latter species, Chatterji and Talpatra (2), isolated reserpine and ajmaline. A survey of literature, however, revealed no phytochemical investigation of *R. decurva*.

EXPERIMENTAL

Preliminary Investigation.—It was found that the total alkaloid content of the roots varied inversely with the diameter of the roots. The results according to the method of Horhammer and Rao (3) are:

Thin roots (less than 1 cm. diameter)	1.4%
Medium roots (1–2 cm. diameter)...	0.55%
Thick roots (2–5 cm. diameter).....	0.25%

A paper chromatographic study revealed the presence of eight alkaloidal spots from which probable presence of serpentine, reserpiline, reserpine, rescinnamine, sarpagine, and reserpinine could be shown by direct comparison with known alkaloids and crude alkaloidal extracts of *R. serpentina*, using the method of Carol, *et al.* (4), and Korzun, *et al.* (5).

Extraction and Preliminary Fractionation.—One Kg. coarsely ground root was percolated with methanol, the methanol was removed under reduced pressure, and the residue extracted with a 5% aqueous solution of acetic acid. The acid solution, after defatting with petroleum ether, was extracted by shaking with chloroform. The residue from chloroform extracts represented the weakly basic alkaloids.

Isolation of Reserpiline and Isoreserpiline.—The benzene soluble portion of the weakly basic alkaloids was chromatographed over 100 Gm. of Merck acid-washed alumina using benzene, chloroform, and chloroform containing 1% methanol as the successive eluants. The chloroform and chloroform-methanol eluates gave residues which show a yellow-green color, changing to purple with Frohde's reagent. The pooled residue was dissolved in chloroform and rechromatographed over acid-washed alumina. The residue from chloroform eluates readily formed crystalline oxalate and phosphate from methanolic solutions. It was purified by repeated crystallizations of the oxalate. The base recovered from the oxalate was identical in all respects to isoreserpiline, first reported from *R. canescens* leaves by Stoll, Hoffmann, and Brunner (6). In agreement with the published data the base melted at 210° and the oxalate melted at 245°. $[\alpha]_D^{25} = -82°$ in pyridine. The I. R. and U. V. absorption spectra were identical to those of the respective reference sample. The alkaloid gave typical color reactions with 2 *N* nitric acid and with Frohde's reagent.

The residue from the chloroform, containing 1% methanol eluates, was also purified through its crystalline oxalate and then reconverted into the base. The amorphous base thus obtained was identical in all respects to reserpiline, isolated for the first time from *R. serpentina* by Klohs and coworkers (7). In agreement with the published data the base was amorphous. The oxalate melted at 250°. $[\alpha]_D^{25} = -15°$. The I. R. and U. V. spectra were identical to that of reserpiline. Equivalent weight by potentiometric determination was 414.

Isolation of Reserpine.—After several attempts to isolate reserpine from the weakly basic alkaloids by the usual procedures (8, 9) which were unsuccessful, a 700-Gm. batch of specially selected thin roots was treated according to the procedure of Forbath (10). The reserpine rich fraction obtained by this method on chromatography over alumina yielded 5 mg. of reserpine. It was identified by comparison on a paper chromatogram with pure reserpine, by mixed melting point with reserpine, and by its ultraviolet absorption spectrum. It gave a yellowish-green color changing to blue with Frohde's reagent, characteristic of reserpine. Paper chromatographic evidence showed a considerable amount of reserpine in the mother liquors.

Isolation of Sarpagine.—After the removal of the weakly basic alkaloids from the acetic acid solution of the alkaloids of *R. decurva*, the acid solution was adjusted to a pH of 7. The large amount of yellow precipitate was collected, dissolved in methanol, and a methanolic solution of oxalic acid added. The insoluble alkaloidal oxalates thus formed were removed by filtration and the clear solution chromatographed over Merck alumina, using absolute methanol as the eluant. The first 500 cc. of eluates, after evaporation and dissolving in a small volume of methanol, deposited a white crystalline material

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The author is indebted to Dr. Arthur E. Schwarting of the University of Connecticut for guidance and allowing use of extraction facilities in the earlier phase of this investigation. Sincere thanks are due to Dr. M. W. Klohs of the Riker Laboratories for preparing the I. R. spectra and for many helpful suggestions. For the material for this study the author wishes to thank Dr. P. N. Mehra of Punjab University, India.

which, after repeated crystallizations from methanol, yielded sarpagine. Sarpagine was isolated for the first time by Stoll and Hoffmann (11). The physical constants of the base were in agreement with the published data for sarpagine. The base melted at 320°. The I. R. and U. V. absorption spectra were identical to those of sarpagine. The equivalent weight determined potentiometrically was 348. This is close to that of raupine of Achelis and Kroneberg (12) which has been shown to be sarpagine with one mole of methanol of crystallization. The alkaloid readily reduced Tollen's and Fehling's solutions.

An additional quantity of sarpagine was obtained from the ether extracts of the aqueous solution of alkaloids from which the sarpagine containing fraction had been precipitated by adjusting the pH to 7, as shown earlier. This aqueous solution was processed for recovery of ajmaline according to the usual procedure (13). The fraction which should have given ajmaline, yielded sarpagine instead. There was a conspicuous absence of ajmaline, as also shown by paper chromatography.

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A Study of the Influence of Gibberellic Acid on the Morphology, Histology, and Biosynthesis of the Glycosides of First-Year's Growth of *Digitalis purpurea* L.*

By MAHMOUD D. SAYED† and JACK L. BEAL

Digitalis plants were treated two times weekly by spraying with gibberellic acid solution, representing 100 p. p. m., for about sixty days. Harvesting was done at approximately weekly intervals. A morphological and histological study of the treated plants was made. In addition, the following quantitative determinations were made: total glycosides, total sugar, digitoxose sugar, crude fiber, and chlorophyll. A marked decrease in surface area and dry weight was observed in the plants treated with gibberellic acid. Some of the treated plants developed a definite elongated stem but did not flower. An increase in total glycosides and total sugars was observed in the treated plants, but there was no significant difference in digitoxose content. An increase in chlorophyll was observed in the treated plants. There was an initial increase in crude fiber content in the treated plants; however, at maturity both the treated and untreated plants showed almost the same content.

DURING the past thirty years a large number of Japanese botanists have been working on growth substances which were first shown to be produced by a rice disease fungus, *Gibberella Fujikuroi* (1). They were isolated and given the name Gibberellins, after the fungus, by a group at the University of Tokyo, notably Yabuta and Sumiki, (2).

About 1952, their work came to the attention, apparently independently, of a group at the Imperial Chemical Industries in Britain and another group at the United States Department of Agriculture. These two groups confirmed the Japanese work and contributed their own findings.

Almost simultaneously the American investigators at the U. S. Department of Agriculture (headed by Stodola) (3), and the British group announced the isolation from cultures of the fungus of an entirely new compound which was given the name gibberellic acid (4). Gibberellic acid differs from the gibberellins of the Tokyo workers in optical rotation, infrared spectra, and certain derivatives (5).

The most typical and striking plant responses to treatment with gibberellic acid are stem elongation (5) and increased cell division (6, 7).

Other responses such as increased dry weight (8), increased enzymatic activity, induction of

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flower formation, and others have been reported by several investigators during the last five years (9, 10, 11).

Lang (12) treated a biennial variety of *Hyoscyamus* with gibberellic acid and found that the plants in long days, bolted and flowered the first year.

Digitalis purpurea L. being a biennial plant and of considerable medicinal importance was chosen for the present study to investigate the influence of gibberellic acid on flowering, as well as its effect on the morphology and histology of the plant, biosynthesis of the glycosides, total sugar content, cellulose, and chlorophyll content.

Fig. 1.—A control digitalis plant.

Fig. 2.—A treated digitalis plant exhibiting stem elongation.

EXPERIMENTAL

An aqueous solution of gibberellic acid,[1] as the potassium salt, representing 100 p. p. m. was prepared and used in spraying the treated plants.

The digitalis plants were started from seed in a greenhouse and then transplanted into pots in March 1957. They were transplanted into the medicinal plant garden of The Ohio State University on May 18, 1957. They were divided into treated and control rows which were arranged alternately. Spraying was delayed until the plants recovered from the transplantation into the medicinal plant garden; spraying was started on June 17 and continued twice weekly until September 5.

Harvesting was done at approximately weekly intervals, from July 17 to September 12. The leaves gathered from both the treated and control groups were separately pooled, and three samples each consisting of five leaves were drawn at random and used for fresh and dry weight determination. The leaf measurements were made on the July 24 sample, see Table I.

Three samples were drawn at random from each harvest, rapidly dried at a temperature of 60°, reduced to No. 60 powder, and saved for glycoside, carbohydrate (total sugar), and crude fiber determinations.

The Influence of Gibberellic Acid on the Morphology and Histology of the Plant

Normally *Digitalis purpurea* does not develop a detectable stem during the first year's growth, Fig. 1.[2] However, some of the plants in the experimental plots treated with gibberellic acid developed a definite stem which was much elongated but they did not flower, Fig. 2. The stem was subjected to a morphological and histological study. Other treated plants did not develop a stem and appeared as illustrated in Fig. 3. The influence of gibberellic acid on the leaves was mainly on their shape and surface area.

The Stem.—The stem developed by some of the treated plants, Fig. 4, is erect, woody longitudinally wrinkled, and very hairy. It measures 25–30 cm. in height and 1.5–2 cm. in diameter. In transverse section, Fig. 5, it shows a relatively wide cortex bounded on the inside by a distinct endodermis and on the outside by a hairy epidermis. The vascular system consists of collateral bundles which are separated by narrow medullary rays, and surround a large parenchymatous pith.

The epidermal cells, Fig. 5, are rectangular or subrectangular with slightly wavy anticlinal walls. In size they vary from 32 μ–40 μ in breadth and 12–16 μ in height. The outer wall is covered with a thin smooth cuticle. In surface view they appear polygonal with slightly wavy outline.

Stomata are few in number, each being surrounded by 3–4 cells of the ranunculacious type. The covering trichomes are numerous. They consist of 3–7 cells. The average length of these hairs is from 330–450 μ. The cortex, Fig. 5, varies in thickness from 10–13 layers of cells. The outermost layers are collenchymatously thickened, gradually passing into thin-walled parenchyma. The endodermis consists of tangentially elongated cells with slightly thickened radial walls. The pericycle, Fig. 5, shows isolated or 3–4 grouped lignified fibers separated by pericyclic parenchyma. The fibers are slightly angular or circular in cross section with varying wide lumen. They are 30–48 μ in diameter and 220–240 μ in length. The phloem is well de-

[1] Gibberellic acid was supplied through the courtesy of Merck and Co., Inc., as the potassium salt.

[2] The pictures in this paper were photographed by the Photography Department, The Ohio State University.

TABLE I.—DATA ON MEASUREMENTS, FRESH AND DRY WEIGHTS OF LEAVES

Sample	Fresh Wt., Gm.[a]		Dry Wt., Gm.[a]		Length of Petiole[b]		Dimensions of Lamina, cm.[b]		Surface area, cm.²[c]	
	T	C	T	C	T	C	T	C	T	C
July 17	33.1	37.5	7.03	8.20	...	...	...	...	...	...
July 24	17.1	34.5	3.50	8.90	...	...	...	...	...	...
July 31	17.8	35.9	4.60	8.48	...	...	...	...	...	...
Aug. 9	19.85	37.5	4.51	9.10	...	...	...	...	...	...
Aug. 23	19.58	28.0	4.50	6.70	...	...	...	...	...	...
Sept. 12	19.60	29.1	4.39	6.57	...	...	...	...	...	...
Average	21.17	33.9	4.75	7.99	7.5	2.5	7.2x3.4	6.6x4.5	44.0	109.5

[a] Average of three samples. [b] Average of 45 leaves. [c] Average of 5 leaves. T—Treated, C—Control.

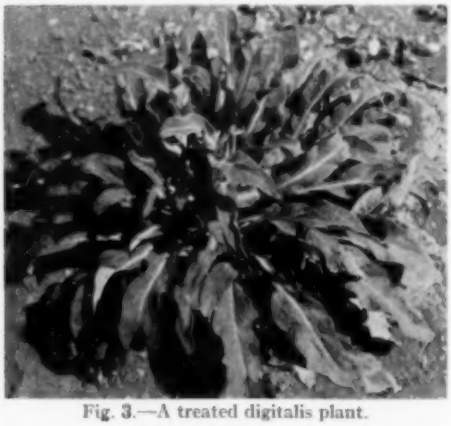

Fig. 3.—A treated digitalis plant.

Fig. 4.—Diagram of a treated, a control leaf and a stem; *T*—leaf of a treated plant; *C*—leaf of a control plant; *St*—stem. × 1/10.

veloped and consists of sieve tubes accompanied with companion cells and intermixed with phloem parenchyma. The wood, Fig. 5, consists mainly of scalariform and spiral vessels varying from 33–78 μ in diameter. The vessels are intermixed with wood parenchyma, tracheids, and wood fibers. The wood fibers are more or less similar in shape to the pericyclic fibers. The pith is composed of large polygonal parenchyma with distinct intercellular spaces and may show simple pits.

The Leaf.—The leaves of the treated plants showed a marked morphological difference from the control, Fig. 4. Their petioles were much longer than those of the control, and their lamina were much more linear and almost lanceolate in shape. For the purpose of comparison, 45 leaves were harvested at random from each group of the July 24 harvest. The length of their petioles, the dimensions of the lamina (length and breadth at the broadest part) were measured to the nearest mm. The results obtained are given in Table I. On studying the histology of the leaves of treated and control plants, no difference could be observed in the type or arrangement of tissues. However, in determining the palisade ratio and vein-islet number, following the procedure described by Youngken (13), some difference could be observed. The results obtained are given in Table II.

TABLE II.—PALISADE RATIO AND VEIN-ISLET NUMBER[a]

	Treated	Control
Palisade ratio	4.2–6	2.8–4
Vein-islet number	3.1–4	6.2–5.8

[a] Average of three determinations of each of three leaves.

The Influence on the Biosynthesis of the Glycosides

Digitalis tinctures were prepared from harvested samples of each group according to the U. S. P. XIV (14). The tinctures were assayed for total glycosides spectrophotometrically following the procedure described by Bell and Krantz (15). The principle of this procedure is based upon spectrophotometric measurement of the color developed by digitalis glycosides on the addition of alkaline picrate solution. The results obtained are expressed as per cent potency in terms of reference standard and are illustrated in Fig. 6.

Influence on Total Sugar Content.—The total sugar content was determined in terms of dextrose following the procedure of the A. O. A. C. (16). The data are presented as per cent in Fig. 7.

Determination of Digitoxose Sugar.—To ascertain information of the influence of gibberellic acid on the biosynthesis of specific sugars of the glycoside molecule, the material was subjected to a quantitative Keller-Kiliani determination to measure the digitoxose present. The procedure followed was that of the U. S. P. XIII (17), method II, for the determination of digitoxin. The determination was done on three samples, notably those harvested on July 17, July 24, and August 23. The results obtained are given in Table III.

TABLE III.—DIGITOXOSE PER CENT

	Digitoxose[a] Per Cent	
	Treated	Control
July 17	0.015	0.015
July 24	0.015	0.015
August 23	0.015	0.009

[a] Average of three determinations.

Crude Fiber Determination.—From a study of Fig. 7, it is observed that the per cent of total sugars is influenced by gibberellic acid. It seemed of interest to study the crude fiber content (as a criterion for cellulose content) of both treated and control plants. The procedure followed was that described in U. S. P. XV (18). The results obtained are given in Table IV.

TABLE IV.—CRUDE FIBER PER CENT[a]

Sample	Crude Fiber Per Cent	
	Treated	Control
July 17	12.10	6.01
July 24	17.20	8.20
July 31	10.01	5.20
August 9	10.30	9.01
August 23	9.40	9.10
September 12	9.42	9.20

[a] Average of three determinations.

Chlorophyll Determination.—Total chlorophyll, chlorophyll a, and chlorophyll b content of the leaves of treated and control plants was determined on three harvests, notably July 17, July 31, and August 23, following the A. O. A. C. method (19) using a Beckman Spectrophotometer, Model DU. The results are given in Table V.

TABLE V.—CHLOROPHYLL PER CENT[a]

	Date of Harvest					
	July 17		July 31		August 23	
	T	C	T	C	T	C
Total chlorophyll	0.295	0.253	0.403	0.234	0.379	0.214
Chlorophyll a	0.198	0.194	0.315	0.167	0.260	0.163
Chlorophyll b	0.094	0.059	0.114	0.051	0.120	0.051

[a] Average of three determinations.
T—Treated, C—Control.

DISCUSSION

A study of some effects of gibberellic acid on *Digitalis purpurea*, has been made. The objectives of this study were to determine its influence on the morphology and histology of the plant and the biosynthesis of the glycosides in the leaves.

The most marked influence on the morphology of the leaf was the decrease in surface area, elongation of the petiole and development of a stem by some of the treated plants.

The shape of the leaf was also influenced, being

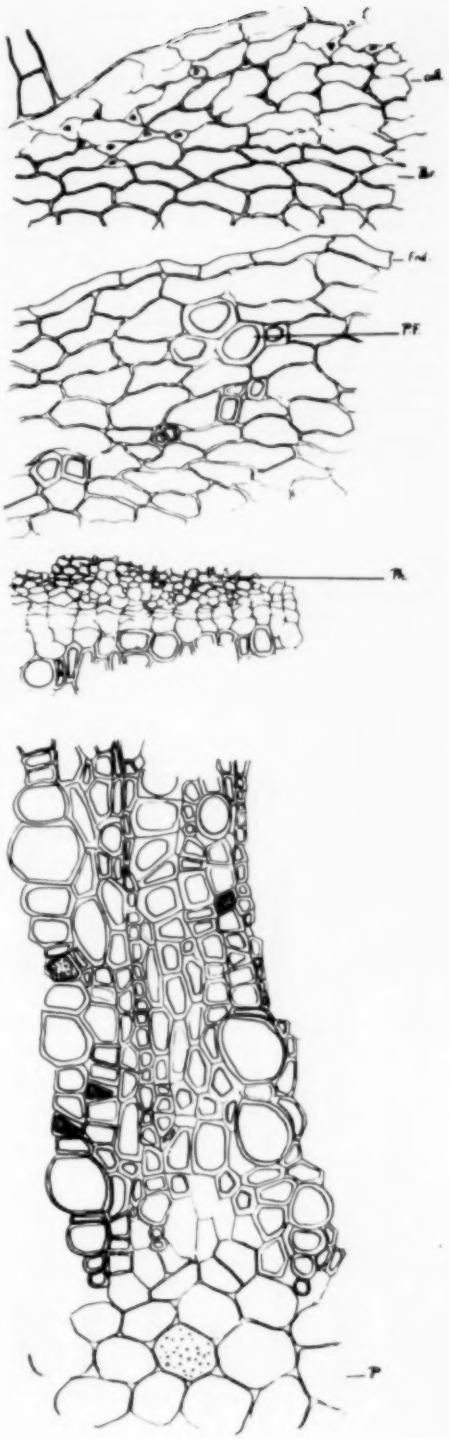

Fig. 5.—Transverse section in the stem; *Coll*—collenchyma; *Par*—parenchyma; *End*—endodermis; *P. F.*—pericyclic fibers; *Ph*—phloem; *P*—pith. × 120.

ovate in the control and almost lanceolate in the treated, Fig. 4. On studying the histology of the leaves, no difference could be observed between both groups. However, some difference could be observed in the vein-islet number and palisade ratio, Table II. The seeming increase in number of palisade cells might partially explain the increase in per cent of carbohydrate in the treated plants since this would mean a larger number of photosynthetic cells per unit area.

A higher glycoside per cent was observed in all harvested samples, Fig. 6, which might suggest either an increase in the aglycone or sugar fraction within the cell. The assay procedure used for the total glycosides is based upon the aglycone fraction of the glycoside molecule. Therefore, an expression of an increase in total glycosides is also an indication of an increase in aglycone and it does not necessarily mean an increase in the sugar fraction, as the latter may occur in the plant either free or in glycosidal form. For this purpose, a determination of total sugars (in terms of dextrose—Fig. 7) and the specific sugar of the glycoside (digitoxose—Table III) was done. These determinations revealed a higher total sugar per cent in treated plants, while digitoxose per cent did not significantly differ in both. This suggests that the increase in glycoside per cent in treated plants may be due to a higher aglycone per cent rather than digitoxose.

On assaying digitalis samples for total glycosides, it was observed that the alcoholic extract of the treated leaves was always emerald green, while that of the controls was greenish brown in color. This difference in color was observed in extracts of all samples representing all harvests. It was decided to carry out a chlorophyll determination of treated and control leaves. Results obtained, Table V, revealed a higher total chlorophyll as well as chlorophyll a and b in treated leaves.

On studying the surface area of both groups, Table I, it was observed that a leaf of the control plant has an average area approximately three times that of the treated. On the other hand, the total carbohydrate determination did not show a similar proportion. It has been reported that the effect of gibberellic acid is not directly related to photosynthesis (20, 21). Thus it could be postulated that the greater surface area of the control plants, requiring more cellulose for cell wall formation, is a possible reason for a lower carbohydrate per cent.

The crude fiber determinations, Table IV, revealed a higher per cent in the treated plants at the earlier harvests. However, the control leaves showed gradual increase in their crude fiber content until almost attaining that of the treated. It could be suggested that this increase in crude fiber per cent of the treated plants at the earlier harvests is due to a faster growth induced by gibberellic acid, in addition to having more elongated petioles.

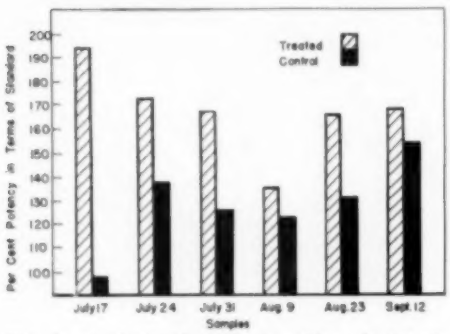

Fig. 6.—Analysis of total glycosides of digitalis samples.

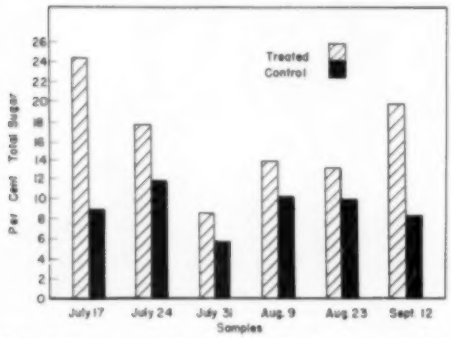

Fig. 7.—Per cent of total sugar in digitalis samples.

CONCLUSIONS

Plants of *Digitalis purpurea*, treated by spraying with a solution of gibberellic acid (100 p.p.m.), showed the following responses:

1. The leaves were morphologically affected, developing longer petioles and less surface area than the control. The shape of the treated leaves was also different in being more linear, almost lanceolate. The dry weight was markedly decreased.

2. Histologically, no difference could be observed between both groups. However, the treated leaves had a higher palisade ratio and a lower vein-islet number.

3. A stem was developed by some of the treated plants.

4. An increase in glycoside and total sugar per cent, but no significant difference could be observed in digitoxose per cent.

5. Crude fiber per cent was almost the same in both groups at maturity; however, the treated plants showed higher content at earlier ages.

6. An increase in chlorophyll per cent, total, a, and b.

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A Study of the Influence of Gibberellic Acid on the Growth of *Fagopyrum esculentum* Moench and the Biosynthesis of Rutin*

By MAHMOUD D. SAYED† and JACK L. BEAL

A study was made of the influence of gibberellic acid on the growth of buckwheat plant and the biosynthesis of rutin. It was determined that gibberellic acid increased the linear growth, dry weight of stems and leaves, number of leaves per plant, total sugar per cent, crude fiber per cent at early stages, and total chlorophyll per cent while a decrease was observed in rutin per cent and rhamnose per cent.

In a previous communication (1) the influence of gibberellic acid on the morphology and the biosynthesis of glycosides of first year's growth of *Digitalis purpurea* L. was presented. In the present investigation, the influence of gibberellic acid on the growth of *Fagopyrum esculentum* Moench (buckwheat) and the biosynthesis of rutin has been studied.

EXPERIMENTAL

Buckwheat plants were propagated from seeds in The Ohio State University College of Pharmacy's medicinal plant garden on May 25, 1957. A group of the experimental plants was treated by spraying them with an aqueous solution containing 100 p. p. m. of gibberellic acid.[1] A similar group was left untreated and used as a control. Treatment was started on June 17, after the true leaves were well developed, and continued twice weekly until September 5. Harvesting was done approximately at weekly intervals from June 28 until September 12. At each harvest, 15 entire plants from each of the treated and control groups were gathered and five plants were further drawn at random from each of the 15 plants. These five plants were used for the determination of the height and dry weight of the stems and dry weight of the leaves. The results obtained are given in Table I.

The leaves of the remainder of gathered plants were dried separately at 105° and three samples were drawn at random from each group and saved for evaluation. When the plants attained maturity (fruiting stage), the number of leaves on 15 plants from each group were counted. The average number of leaves was found to be 118 and 97 leaves per plant in the treated and control groups, respectively.

For rutin estimation the spectrophotometric procedure of Arthur (2) was followed. The principle of this procedure is based on spectrophotometric measurements of rutin aluminum chloride complex which has an absorption maximum at 416 mμ. This rutin complex is stable, and the absorbance remains unchanged for at least two hours. Thus all determinations were done within this period. The results obtained are given in Table II.

Total sugar was determined in terms of dextrose following A. O. A. C. (3) procedure. The results obtained are illustrated in Table II.

From a study of Table II it may be observed that the percentage of rutin in treated plants is less, while the percentage of total sugar is more than the controls. It was deemed pertinent, therefore, to study the influence of gibberellic acid on the specific sugar rhamnose which is necessary for the biosynthesis of the glycoside.

Separation of rhamnose was carried out by paper chromatography. The buckwheat samples were extracted with 80% alcohol and the extract purified following the A. O. A. C. procedure of total sugar analysis (3). An aliquot portion of the clear extract was spotted on Whatman No. 1 filter paper. In addition to the buckwheat extract the following chromatograms were run simultaneously: buck-

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[1] Gibberellic acid was supplied through the courtesy of Merck and Co., Inc., as the potassium salt. The product is known as Gibrel.

TABLE I.—DATA ON LENGTH OF STEMS, DRY WEIGHTS OF STEMS, AND LEAVES OF BUCKWHEAT SAMPLES

Date of Harvest	Length of Stem, cm.		Dry Wt. of Stem, Gm.[a]		Dry Wt. of Leaves, Gm.[a]	
	T	C	T	C	T	C
June 28	50.8	34.1	5.3	2.5	4.8	3.4
July 10	71.5	56.0	6.2	3.9	6.5	4.5
July 17	82.4	52.2	7.4	3.2	6.06	3.3
July 24	87.2	55.2	13.4	3.8	10.6	3.3
July 31	91.1	63.2	26.4	15.3	15.1	11.2
Aug. 23	77.1	51.9	15.8	5.7	9.5	6.3
Sept. 12	72.2	54.8	14.8	6.3	9.3	6.1

[a] Average of 5 samples, dried at 105°. T—Treated, C—Control.

TABLE II.—PERCENTAGE OF RUTIN, TOTAL SUGARS, RHAMNOSE, CRUDE FIBER, AND CHLOROPHYLLS IN BUCKWHEAT SAMPLES[a]

Date of Harvest	Rutin, %		Total Sugars, %		Rhamnose, %		Crude Fiber, %		Chlorophyll, %	
	T	C	T	C	T	C	T	C	T	C
June 28	4.10	4.30	3.21	2.97	1.30	1.50	1.27	0.81	3.60	2.56
July 10	3.83	4.88	6.68	3.06	..	..	0.98	0.90	..	..
July 17	3.41	4.40	6.63	3.34	..	..	0.81	0.88	..	..
July 24	3.71	4.27	6.46	3.49	2.56	2.78	0.73	0.98	2.36	2.24
July 31	2.34	3.60	5.51	4.14	..	..	0.81	1.03	..	..
Aug. 23	2.70	3.31	4.99	4.38	2.41	2.83	1.03	1.01	3.03	2.72
Sept. 12	2.68	3.26	4.91	4.19	..	..	1.06	1.05	..	..

[a] All are averages of three determinations. T—Treated, C—Control.

wheat extract plus rhamnose, rhamnose, glucose, and rhamnose plus glucose.

The solvent system used was ethyl acetate-pyridine-water (8:2:1). The chromatograms were equilibrated for fifteen hours in chambers previously saturated with the solvent, and were formed in a descending direction. After five hours of chromatographing, the strips were air-dried and sprayed with aniline hydrogen phthalate reagent and then heated in an oven from five to ten minutes. The separated rhamnose was identified by the color reaction (red), by the R_f value compared with that of pure rhamnose, as well as by mixed chromatograms (plant extract and rhamnose) run under identical conditions. Other sugars appeared on the chromatograms prepared from the plant extract which were not identified, since none of them interfered with rhamnose. The amount of rhamnose was estimated spectrophotometrically following the procedure described by Loewus (4) which is based on the spectrophotometric determination of the color produced by rhamnose on the addition of a 2% anthrone solution in ethyl acetate in the presence of sulfuric acid.

Rhamnose was recovered from the chromatogram by carefully packing that portion of the paper strip carrying this sugar in a thimble and extracting with 80% alcohol. The average percentage recovery was found to be 87%.

Rhamnose determination was done on three harvests, notably June 28, July 24, and August 23. The results obtained are given in Table II, which shows that the percentage of rhamnose is significantly higher in the control than in treated leaves. These values were significant at the 5% level using the Student "t" test with four degrees of freedom.

In order to have a more complete idea as to the nature of the limiting factor causing a decrease in rutin concentration in the treated plants, an examination was made to determine the amount of free quercetin. For this purpose samples of buckwheat weighing approximately 3 Gm. representing different stages of age of the plant, namely thirty-four, sixty-seven, and ninety days, were extracted with absolute alcohol in a Soxhlet extractor. The alcoholic extract in each case was concentrated by spontaneous evaporation to about 10 ml. About 200 µl. was spotted on strips of Whatman No. 1 filter paper. The following solvent systems were tried: chloroform-*n*-butanol-water (2:4:4) v/v; acetic acid-water (6:4) v/v; ethyl acetate–acetic acid–water; and *n*-butanol–acetic acid–water (4:1:5) v/v.

In each solvent system, control chromatograms of pure rutin, pure quercetin, and a mixture of both pigments were run in addition to the buckwheat extract. The chromatograms were equilibrated with the particular solvent for fifteen hours and descendingly run for six hours. Rutin and quercetin, when present were identified by their R_f values, the color of their respective aluminum chloride complex, and the fluorescence of this complex under the ultraviolet light. Rutin gives a yellow, while quercetin gives an emerald green fluorescence. The chromatograms were air-dried, sprayed with 0.1 *M* aluminum chloride solution, and examined under the ultraviolet light. No free quercetin could be detected in the buckwheat extract at any time while, with each system, rutin was detected. Table III lists the R_f values of the rutin control and the quercetin control in the different solvent systems.

TABLE III.—R_F OF RUTIN AND QUERCETIN[a]

	$CHCl_3$–BuOH–H_2O	AcOH–H_2O	EtAc–AcOH–H_2O	BuOH–AcOH–H_2O	EtAc–AcOH–H_2O
Quercetin	0.05	0.82	0.78	0.75	0.92
Rutin	0.52	0.72	0.11	0.51	0.11

[a] Average of three determinations.

To study the influence of gibberellic acid on the cellulose content of buckwheat during the growing season, quantitative crude fiber determinations were carried out following the procedure of the U. S. P. XV (5). The results obtained are given in Table II.

Three harvests of treated and control buckwheat leaves, June 28, July 24, and August 25, were subjected to a quantitative chlorophyll determination, following the procedure of A. O. A. C. (6). Table II illustrates the results obtained.

DISCUSSION

The morphological response of buckwheat plants was observed to be mainly in linear growth and an increase in dry weight. No observed difference could be detected in the shape or size of the leaves of the experimental plants; however, the treated plants averaged more leaves per plant than did the controls. The increase in dry weight of stems and leaves was of such magnitude that it could not be accounted for by the difference in the number of leaves per plant nor by the increase in cell wall that results from mere cell elongation. However, increased cell division could bring about an increase in dry weight due to increase in cell wall formation and increase in cell contents due to the presence of more cells. The formation of new cells involves not only the division of pre-existing cells but the subsequent enlargement and maturation of their cell progeny. The determination of the cellulose content (crude fiber content) of treated and control plants at periodic intervals reveals an increase in cellulose content of treated plants, particularly in early harvests. This could possibly suggest that gibberellic acid stimulates faster cell division, and thus cell maturation is attained earlier than control plants. By the time the control plants reach a comparable degree of maturity as the treated, at later harvests, the cellulose content of both groups becomes almost the same.

Although treated and control plants showed almost the same cellulose content at the latest harvest, the dry weight of the former remained higher. This suggests that increased cell wall formation owing to cell division in treated plants cannot solely be responsible for the increased dry weight. Determination of total carbohydrates Table III, revealed an increase in treated plants in all harvests, which could also mean an increase in dry weight.

It has been reported that gibberellic acid has no direct effect on photosynthesis (7, 8). Thus, the increase in total sugar induced by gibberellic acid is most likely due to an indirect factor related to photosynthesis output. It has been observed that there is a marked increase in the number of leaves per buckwheat plant in the treated groups, which means an increase in the photosynthetic area. Since the carbohydrates formed during photosynthesis are translocated to all parts of the plants, it could be suggested that an increase in total carbohydrate per cent is to be expected in treated plants owing to the increased number of leaves.

The glycoside percentage was always relatively lower in treated plants reaching a peak at a later date than controls. However, if the rutin content had been calculated on dry weight basis of leaves per plant, the treated leaves would have shown a higher content than controls. This is due to the relative increase in the dry weight and number of leaves of treated plants.

It was observed that the control plants had a higher rhamnose percentage than the treated plants. Thus the decrease in rutin content in treated plants could possibly be due to either of the following factors: effect induced by gibberellic acid on the enzyme or enzymes responsible for linking the sugar fraction with the aglycone fraction of the glycoside; less available rhamnose sugar in treated plants; or less available aglycone (quercetin) in treated plants which could be due to an influence of gibberellic acid at a stage in the aglycone biosynthesis.

If the influence of gibberellic acid has a retarding effect on the activity of the enzyme or enzymes responsible for linking the sugar fraction with the aglycone fraction, without influencing the quercetin formation, the latter would have occurred, at least in part, free in the plant. However, this was not the case, since no free quercetin could be detected in the experimental plants. This suggests that the influence of gibberellic acid is most probably at a stage of quercetin biosynthesis, or on rhamnose formation, or both together.

CONCLUSIONS

Plants of *Fagopyrum esculentum* Moench treated with a solution of gibberellic acid (100 p. p. m.) showed the following responses:

1. An increase in linear growth and dry weight of stems. As well as an increase in dry weight of the leaves.
2. An increase in the number of leaves per plant.
3. A decrease in rutin per cent
4. An increase in total sugar per cent.
5. A decrease in rhamnose sugar per cent.
6. Crude fiber was almost the same at maturity, however, the treated plants showed higher content at earlier ages.
7. An increase in total chlorophyll per cent.

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The Antisecretory and Antipeptic Activity of Gastric Antacids in the Histamine-Stimulated Rabbit*

By E. W. PACKMAN, D. D. ABBOTT, B. TRABIN, and J. W. E. HARRISSON

The effects of dihydroxy aluminum sodium carbonate (DASC), aluminum hydroxide, calcium carbonate, and sodium bicarbonate, upon the proteolytic activity and volume of gastric secretion of the histamine-stimulated, pyloric-ligated rabbit have been studied. The probable clinical importance of the several factors that were observed has prompted the formulation of an "Index of Effectiveness" to be used as a means of evaluating gastric antacids. This index employs four factors: the maintenance of pH within the range of 3 to 5.5, *in vitro* acid consuming power, proteolytic activity, and the volume of the gastric secretion. Using this index, the antacids in order of decreasing effectiveness were: dihydroxy aluminum sodium carbonate, calcium carbonate, sodium bicarbonate, and aluminum hydroxide.

GASTRIC ANTACIDS as therapeutic agents are primarily employed in the clinical treatment of hyperacidic conditions and peptic ulcers. Since excessive secretion of gastric juice is considered to be one of the prime factors in the formation of peptic ulcers (1) and indigestion or "heartburn," it is quite understandable that antacid agents which may neutralize this excess acidity, afford the patient some symptomatic relief. Many patients receiving antacid therapy experience much more relief than can be attributed to the chemical neutralizing power of the antacid. With this in mind, several investigators have studied experimentally the effects of antacids upon the volume of gastric secretion and pepsin activity, since these factors may also play an important role in the pathogenesis of peptic ulcer. Komarov and Komarov (2), Komarov and Kruegar (3), and Schiffrin and Komarov (4) have demonstrated in animals that aluminum hydroxide not only diminished the acidity of the gastric juice but also inactivates pepsin. However, Clark and Adams (5) found that aluminum hydroxide had no effect upon the volume of gastric secretion in the Cope-pouch dog. From the same study these investigators concluded that sodium bicarbonate and calcium carbonate actually increased the volume of gastric secretion in the test animal. More recently the early work upon aluminum hydroxide of Komarov, *et al.* (2, 3, 4), was confirmed by Rosen, *et al.* (6), in the pyloric-ligated rat.

In previous investigations, in our own laboratory, we have evaluated the neutralizing or antacid properties of four well-known compounds, dihydroxy aluminum sodium carbonate (DASC), aluminum hydroxide, calcium carbonate, and sodium bicarbonate. These studies have included observation of the changes in gastric acidity produced in the Pavlov-Fistula-dog (7) and in man (8), as well as the degree of protection afforded by these antacids against histamine-induced ulceration in guinea pigs (9, 10). Until now, we have not determined the effect of these agents upon the volume and proteolytic activity of gastric secretion.

We have, therefore, planned this study toward evaluating the effect of the aforementioned antacids upon the volume and proteolytic activity of the gastric secretion. Preliminary work indicated that the rabbit would be an appropriate animal, although earlier literature references seemed to contradict its usefulness. To insure "complete" collection and adequate volume of gastric juice, we employed a pyloric-ligated preparation stimulated with histamine.

In addition, we have evaluated the effect of these antacids upon proteolytic activity *in vitro*.

METHODS

In vivo Gastric Secretion and Proteolytic Activity.—Healthy, male, albino rabbits, weighing between two and four Kg. were employed throughout this study. Each animal was fasted thirty-six hours previous to the experiment, but water was supplied *ad libitum*. Anesthesia was induced by intravenous injection of 35 mg./Kg. pentobarbital sodium in aqueous solution. The abdominal area was shaved and thoroughly cleansed with antiseptic and a 3-inch vertical abdominal incision made. The stomach was exposed, examined to note any abnormalties, and finally the pylorous ligated with silk thread. A small incision was then placed in the esophagus and a small caliber polyethylene tube inserted through this incision down into the stomach. The stomach contents, prior to beginning the experiment, were aspirated via this tube and the tube left in place, in order to prevent any excess pressure build-up within the stomach.

An intramuscular injection of 0.25 mg./Kg. histamine diphosphate was made after the stomach had been ligated and "emptied" by aspiration. Immediately following the histamine, 0.5 Gm. of antacid suspended or dissolved in 5 ml. distilled

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water was administered into the "empty stomach" via the polyethylene tube in the esophagus. Control animals received 5 ml. of distilled water following histamine injection. At the end of sixty minutes, the stomach was removed, the stomach contents collected and strained through absorbent cotton, and the volume, pH, free titratable acidity, and proteolytic activity determined, employing a hemoglobin substrate.

In Vitro Neutralization and Protective Activity.—One hundred milliliters of rabbit gastric juice were collected from a pyloric-ligated rabbit receiving histamine. This juice was divided into four separate 25-ml. samples. One of the following antacids was added to each 25-ml. sample: 500 mg. aluminum hydroxide, 500 mg. calcium carbonate, 500 mg. sodium bicarbonate, and 500 mg. DASC.

The samples were stirred constantly, incubated at 37° for one hour, and subsequently pH, free titratable acidity, and proteolytic activity were determined upon the strained samples.

RESULTS

The data regarding the effect of dihydroxy aluminum sodium carbonate (DASC), aluminum hydroxide, calcium carbonate, or sodium bicarbonate upon acidity and volume of gastric secretion induced by histamine and the proteolytic activity of this secretion are presented in Tables I–V and Figs. 1–4. Tables I–IV comprise the data upon fifteen different animals, while table V contains data upon twenty different animals in the control group.

In vitro buffering or neutralizing activity of the four antacids studied upon rabbit gastric juice is presented in Table VI.

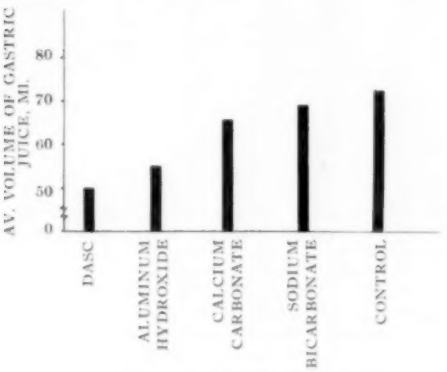

Fig. 1.—Effect of antacids upon the volume of gastric secretion in the pyloric-ligated rabbit.

DISCUSSION

We have found that each of the four antacids which were studied decreased the volume of gastric secretion. These results are presented in Fig. 1.

From the preceding data, it is evident that both DASC and aluminum hydroxide exert greater "antisecretory" effect than do calcium carbonate or sodium bicarbonate. The exact mechanism by which this action is manifest cannot be explained at this time. There are, however, several avenues of thought on this subject.

TABLE I.—EFFECT OF DASC UPON GASTRIC SECRETION OF THE PYLORIC-LIGATED RABBIT

Total Volume	pH	Free Acid/100 ml.	Mg. Pepsin/ml.	Total Chlorides, Gm./100 ml.
47	3.3	None	1.7	0.51
53	2.9	10.7	2.2	0.49
64	2.5	25.7	4.1	0.48
54	2.29	20.3	2.1	0.52
52	3.18	None	1.8	0.48
67	3.24	None	2.8	0.54
41	2.16	25.7	3.4	0.54
40	3.09	None	2.1	0.56
51	2.55	10.7	4.3	0.52
45	2.71	None	2.2	0.53
30	4.4	None	1.8	0.48
39	2.8	None	2.4	0.46
55	3.1	None	0	0.51
64	5.5	None	1.9	0.57
58	5.0	None	0	0.51
		Range		
30–67	2.16–5.5	0–25.7	0–4.3	0.46–0.57

TABLE II.—EFFECT OF ALUMINUM HYDROXIDE UPON GASTRIC SECRETION OF THE PYLORIC-LIGATED RABBIT

Total Volume	pH	Free Acid/100 ml.	Mg. Pepsin/ml.	Total Chlorides, Gm./100 ml.
54	1.7	64.1	2.8	0.41
65	1.7	44.9	3.3	0.42
62	1.85	59.9	4.9	0.46
65	1.4	94.1	1.9	0.44
43	1.8	47.0	4.4	0.37
80	1.7	55.6	1.8	0.48
57	1.5	57.7	3.0	0.47
38	1.8	40.6	3.1	0.52
60	1.2	79.1	1.4	0.51
67	1.9	32.1	1.4	0.41
42	1.1	83.3	1.3	0.49
50	1.5	66.3	2.3	0.46
46	1.7	51.3	1.8	0.42
44	1.8	41.7	3.4	0.51
51	1.5	47.1	2.6	0.47
		Range		
42–80	1.1–1.9	32.0–94.1	1.3–4.9	0.37–0.52

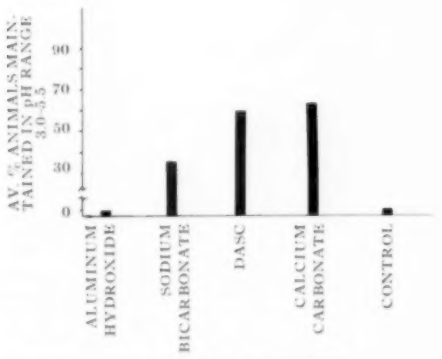

Fig. 2.—Ability of antacids to maintain pH of gastric secretion in range 3.0–5.5.

TABLE III.—EFFECT OF CALCIUM CARBONATE UPON GASTRIC SECRETION OF THE PYLORIC-LIGATED RABBIT

Total Volume	pH	Free Acid/100 ml.	Mg. Pepsin/ml.	Total Chlorides, Gm./100 ml.
52	3.2	None	3.1	0.57
48	4.6	None	2.6	0.59
45	4.6	None	3.5	0.54
80	5.2	None	4.7	0.56
68	3.9	None	1.9	0.57
92	2.5	None	2.3	0.58
58	4.3	None	2.5	0.48
65	1.4	56.7	1.6	0.53
71	2.75	None	1.6	0.56
61	5.21	None	2.3	0.53
75	1.4	57.7	1.5	0.53
68	4.0	None	1.1	0.55
87	3.5	None	0.5	0.52
60	1.7	66.3	1.7	0.49
65	2.6	15.8	1.4	0.53
		Range		
45–92	1.4–5.21	0–66.3	0.5–4.7	0.48–0.59

TABLE IV.—EFFECT OF SODIUM BICARBONATE UPON GASTRIC SECRETION OF THE PYLORIC-LIGATED RABBIT

Total Volume	pH	Free Acid/100 ml.	Mg. Pepsin/ml.	Total Chlorides, Gm./100 ml.
57	1.35	57.7	1.5	0.55
68	4.0	None	1.1	0.53
47	3.5	None	0.5	0.52
68	5.0	None	0	0.52
79	1.4	62.3	0.6	0.54
63	1.1	111.7	2.3	0.56
55	3.1	None	0	0.53
64	5.5	None	1.9	0.55
93	7.5	None	1.2	0.54
70	1.8	23.5	2.4	0.55
83	6.7	None	0	0.52
85	7.2	None	0	0.52
63	1.1	111.7	2.3	0.54
79	1.4	62.3	0.6	0.56
72	1.0	87.6	2.1	0.56
		Range		
47–93	1.0–7.5	0–111.7	0–2.3	0.52–0.56

TABLE V.—GASTRIC SECRETION OF THE PYLORIC-LIGATED RABBIT

Total Volume	pH	Free Acid/100 ml.	Mg. Pepsin/ml.	Total Chlorides, Gm./100 ml.
85	1.4	74.8	5.0	..
97	1.0	117.6	2.3	..
94	1.0	121.8	3.7	..
75	1.1	84.5	1.7	0.53
72	1.0	117.6	2.1	0.56
65	1.2	81.6	1.3	0.50
66	1.3	85.9	2.3	0.51
53	1.6	51.3	2.4	0.48
45	1.3	85.5	1.8	0.44
54	2.0	32.1	1.6	0.26
50	1.7	66.3	1.7	0.49
60	1.3	100.5	3.5	0.50
99	1.2	98.4	3.6	0.51
61	1.2	57.7	1.9	0.48
69	1.5	34.7	0.9	0.48
87	1.4	71.6	2.6	0.51
74	1.1	96.2	3.2	0.49
90	1.5	62.0	4.6	0.51
88	1.2	78.0	2.9	0.48
91	1.1	111.2	1.9	0.54
		Range		
45–99	1.0–2.0	32.1–121.8	0.9–5.0	0.26–0.56

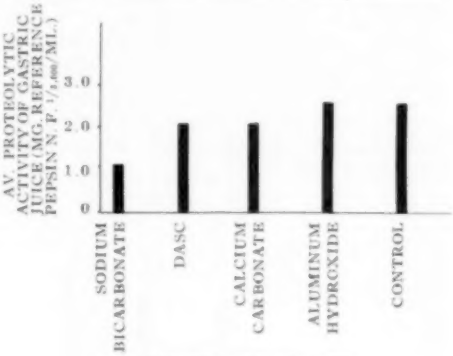

Fig. 3.—Effect of antacids upon the proteolytic activity of gastric secretion in the pyloric-ligated rabbit.

It is generally accepted that the aluminum ion exhibits an astringent effect upon mucous membranes, therefore, if this astringency were to manifest itself upon the gastric mucosa, it is conceivable that secretions from the parietal cells would be diminished. Both DASC and aluminum hydroxide contain aluminum and it is these two antacids which have exhibited the greatest antisecretory activity in this study. This effect was reported several years ago by Babkin and his associates (11).

In addition, if one assumes that the gastric-secretion stimulating effect of histamine is illicited through the release of the gastric hormone, gastrin, adsorption or physical binding of this hormone to some degree by an antacid, could result in a decrease in gastric secretion. Of the four antacid agents investigated, only DASC and aluminum hydroxide may be expected to exhibit the surface phenomenon of adsorption.

If we review critically the effect of the four antacids upon the acidity of the gastric secretions in the pyloric-ligated rabbit, we find that calcium carbonate and DASC maintain the pH range of 3 to 5.5 far better than the other two antacids studied. Data presented in Fig. 2 summarizes the "ability" of these antacid agents to maintain pH in the "safe zone."

All of the antacids were administered in a dosage of 0.5 Gm. It has been previously shown (6) that large increases in dosage will not materially alter the buffering ability of DASC; however, calcium carbonate and sodium bicarbonate in higher dosages will raise the pH of the gastric contents above the "safety-zone" (pH 3–5.5) and sometimes the pH will rise above pH 7.

The third criterion which was evaluated as a measure of effectiveness was in *in vivo* effect upon proteolytic activity of the gastric secretions of the pyloric-ligated rabbit. The reduction in proteo-

TABLE VI.—*In Vitro* EXPOSURE OF RABBIT GASTRIC JUICE (HISTAMINE-STIMULATED) TO ANTACIDS FOR ONE HOUR AT 37°*

Sample	pH	Free Acid/ 100 ml.	Combined Acid/ 100 ml.	Mg. Pepsin/ml.
Pooled control	1.22	84.45	40.62	4.8
25 ml. control, 500 mg. $Al(OH)_3$	2.76	21.38	83.38	3.4
25 ml. control, 500 mg. $CaCO_3$	6.25	...	12.83	0.4
25 ml. control, 500 mg. $NaHCO_3$	7.85	...	11.76	0
25 ml. control, 500 mg. DASC	4.18	...	49.17	2.1

* All analyses were made upon samples which had been strained through absorbent cotton.

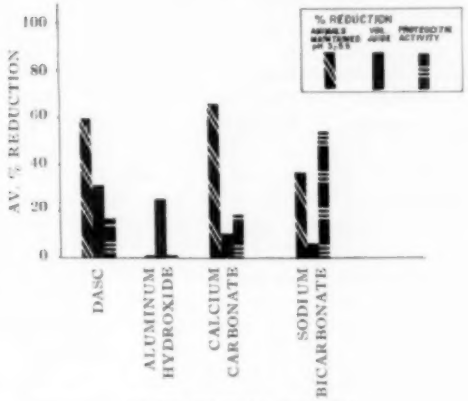

Fig. 4.—Comparative overall effectiveness of antacids.

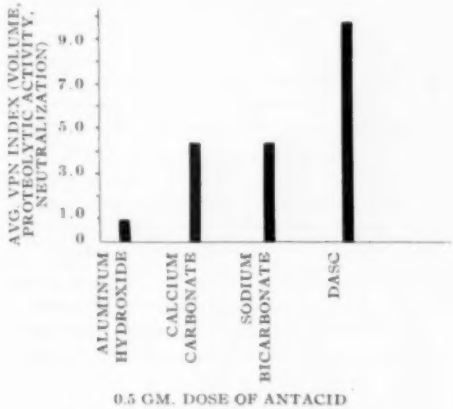

Fig. 5.—VPN Index of antacids studied.

lytic activity which we have observed supports the premise that this decrease in proteolytic activity is due for the most part to an alteration (rise) in pH of the gastric contents and not to a direct effect upon the proteolytic enzymes. Proteolytic activity of the gastric contents following administration of 0.5 Gm. of antacid is presented in Fig. 3.

It is apparent that depending upon which criteria of evaluation one chooses, *i. e.*, reduction in volume of secretion, maintenance of pH range 3-5.5, or decrease in proteolytic activity, the effectiveness of individual antacid agents varies greatly. We have devised a formula, termed the VPN Index, which combines the three criteria previously described into one term covering overall effectiveness of the antacids.

This index may be calculated as follows:

$$\text{VPN Index} = \frac{\text{Reduction in gastric secretion, \%} \times 1 + \left[\text{Reduction in proteolytic activity, \%} \times \text{Animals maintained in pH range 3-5.5, \%}\right]}{\text{Acid consuming power}}$$

in which V = volume, P = proteolytic activity, N = neutralizing effect; and acid consuming power is determined by the U. S. P. procedure described under the monograph specifically for aluminum hydroxide.

Employing this method of combining the data, we obtain the following VPN Indexes for the four antacids studied: DASC, 9.8; calcium carbonate, 4.4; sodium bicarbonate, 4.3; and aluminum hydroxide, 1.0 (Fig. 5).

The VPN Index, as we have calculated it, takes into consideration not only the *in vivo* criteria measured in our experiments, but also the relative *in vitro* acid consuming power of each antacid. This latter consideration of acid consuming power makes the comparison of the effectiveness of these agents relatively more equivocal since we have employed equal dosages of each agent. Furthermore, the VPN Index expresses more completely the true physiologic value of an antacid compound.

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Comparative Rate of Absorption of Four Commercial Analgesic Tablets*

By J. W. E. HARRISSON, E. W. PACKMAN, and D. D. ABBOTT

The degree of absorption at various time intervals of acetylsalicylic acid and acetophenetidin following the administration of tablets containing these substances, varies within the individual subject from day to day as widely as it does between different subjects. This variation in response between tablets can be influenced by differences in their physical properties or composition; however, it is also probable that the great differences which do occur may be due to the widely varying physiologic state of the subject.

DURING the last half century almost 5,000 analgesic or pain relieving drugs have been introduced into the practice of medicine. However, in actual practice the compounds which have enjoyed the greatest favor have been the salicylates, and more specifically acetylsalicylic acid, commonly known as aspirin. Although several tons of aspirin are consumed daily, the exact mechanism of its pain relieving action remains obscure. Moreover, the total plasma salicylate level following oral ingestion of salicylate has been a controversial point over the years. Many investigators have reported the presence of salicylates in the blood within ten minutes after oral ingestion of ten grains of aspirin. Since, it is generally accepted that stomach emptying time is greater than ten minutes, it may be assumed that some degree of absorption must occur before the drug is expelled from the stomach into the small intestines.

Bradley and his co-workers (1) and Carnot and his associates (2) have demonstrated the presence of salicylate in the blood following administration to pylorus ligated dogs. The latter investigators observed these blood levels within three minutes after administration, while Blume and Nohara (3) detected salicylate in the blood of rabbits one and one half minutes after administration. Bradley, *et al.* (1), have also shown that the absorption of salicylates from the upper gastrointestinal tract may be altered by changes in the pH of the stomach contents, which in turn may be related to the emptying time of the stomach. This observation is exemplified by the result of Smull, *et al.* (4), and Parker (5), which indicated that during salicylate administration, the addition of sodium bicarbonate to the mixture caused a marked reduction in plasma salicylate concentration. Recently, there has been much controversy over the plasma salicylate levels resulting from administration of aspirin with buffering agents. Paul, *et al.*(6), has published data indicating higher plasma salicylate levels following oral ingestion of "buffered aspirin" than from aspirin alone at ten minutes and twenty minutes. However, in contrast, Baterman, *et al.*(7), has found no such difference in absorption of buffered and unbuffered aspirin. An extensive review of the literature prior to 1949 was made by Smith (8), to which one may refer for numerous references.

In view of this conflicting data, it was our intention to investigate the extent to which the widely available salicylate containing commercial tablets differ with respect to their absorption as measured by the plasma salicylate or blood *p*-aminophenol levels.

Four brands of analgesic tablets obtained in the market, all of which contained aspirin, and two of which contained acetophenetidin (A. P. C.) were compared. (See Table I.) These preparations were as follows:

TABLE I.—COMPOSITION OF TABLETS

Tablet A...	Aspirin (4 grains), acetophenetidin ($2^1/_2$ grains), and caffeine.
Tablet B...	Buffered aspirin (5 grains) and, in addition, contains magnesium carbonate and dihydroxy aluminum amino acetate.
Tablet C...	Aspirin (5 grains).
Tablet D..	Aspirin ($3^1/_2$ grains), acetophenetidin (3 grains), and caffeine.

It cannot be denied that comparison would have been easier if each product had contained an identical amount of each medicament. However, as commercially distributed products were employed, this ideal of equal dosage of all ingredients was not attainable.

METHODS

Subjects.—Fifteen healthy males between the ages of twenty-one and thirty-five were employed

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in this study. Individual experiments were conducted in the early morning before breakfast on a fasting stomach. Approximately half of this group eventually received each product twice, when it became evident that variation within individuals appeared to be a factor.

Administration of Products.—Two tablets of each of the four products were personally administered to each of the subjects on a given day. These tablets were swallowed with the aid of 60 ml. of tap water (at room temperature). On successive experiments the products were rotated among the subjects, so that each subject received each product at least once, and some twice, during the study. A period of at least seven days elapsed between the administration of the tablets in any run. This time spacing was desirable because of the number of venous punctures and the relatively large total volume of blood that was withdrawn each time. There were three experimental runs which will be commented upon later.

Sampling Intervals.—Forty ml. of blood were removed "one minute" after ingestion of the tablets. This was accomplished by applying the tourniquet forty-five seconds after tablet ingestion and withdrawing the blood sample over a period of one minute. Thus, the "one minute" sample is withdrawn between forty-five seconds and one minute, forty-five seconds after the tablets were swallowed. Thereafter, 20-ml. samples were withdrawn at eight, twenty-five, sixty, and, in later runs, at one hundred twenty minutes after ingestion of the tablets. Previous to the administration of a medicament, a "zero" time sample had also been drawn, which is not reported in the text. All samples were collected in tubes containing heparin. Total plasma salicylate was determined following administration of each brand of aspirin tablets (tablets B and C), and total plasma salicylate, as well as total conjugated blood *p*-aminophenol after administration of each brand of A. P. C. tablets (tablets A and D).

Methods of Analysis.—Total plasma salicylate was determined by a modification of the spectrophotometric method of Brodie, *et al.* (9); using an increased volume of plasma and a double extraction by the solvent. Total conjugated *p*-aminophenol (a measure of acetophenetidin absorption) was determined by the procedure described by Brodie and Axelrod (10).

Experimental Runs and the Results.—Originally the collaboration of seven subjects was obtained who were willing to submit to the five intravenous punctures at rapid time intervals which drew nearly 150 ml. of blood. This schedule was repeated upon these subjects every week for four weeks to cover the administration of all four tablets. There were three experimental runs, each of which was undertaken when it was noted that the data in the completed run suggested a somewhat new approach.

Run A.—The data from this group are tabulated as Run A in Table II for acetylsalicylic acid and Table V for *p*-aminophenol. The subjects are identified as 1a to 7a.

Run B.—Upon reviewing the results obtained in Run A and noting the rapid absorption in some individuals of some products, within "one minute," an additional group of eight different subjects was assembled to confirm this rapid absorption. A one hundred twenty minute time period after administration was also introduced in order to establish, if possible, the declining point in acetylsalicylic acid and acetophenetidin concentration. All four tablets were administered and the plasma salicylate determined only at the one minute and at the one hundred twenty minute time intervals. The "one minute" period was repeated in order to confirm the early absorption of acetylsalicylic acid and the longer period was introduced to establish, if possible, the declining point. In addition, all the new subjects on the A. P. C. tablets (tablets A and D) were sampled at the eight, twenty-five, and sixty minute period for the determination of both plasma salicylate and blood *p*-aminophenol,

TABLE II.—RUN A, TOTAL PLASMA SALICYLATE, μg./ml.

Tablet[a]	Subject 1a	2a	3a	4a	5a	6a	7a	Average
				One Minute				
A	1.3	0.0	0.0	0.3	0.3	1.4	0.1	0.5
D	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
B	0.0	0.0	0.0	0.1	1.3	0.0	0.0	0.2
C	0.0	0.2	0.0	0.0	1.5	0.7	0.4	0.4
				Eight Minutes				
A	4.2	1.0	0.6	2.0	3.2	2.2	3.1	2.3
D	0.0	2.2	0.0	2.8	1.0	1.6	1.0	1.2
B	4.5	4.4	0.0	7.5	5.1	15.8	2.8	5.7
C	1.1	0.4	4.6	3.9	2.9	5.4	8.0	3.8
				Twenty-five Minutes				
A	7.5	7.0	5.5	5.8	23.6	8.3	14.9	10.4
D	3.0	6.1	0.5	3.0	3.0	2.2	6.6	4.3
B	18.0	20.0	7.6	15.8	10.9	35.4	14.2	17.4
C	7.1	13.1	19.2	23.6	24.8	9.3	11.4	15.5
				One Hour				
A	19.2	33.4	9.5	20.6	33.6	13.9	27.6	22.5
D	14.6	18.0	1.7	11.0	5.8	17.2	7.4	10.8
B	37.1	37.4	24.0	20.0	24.9	31.3	3.8	25.5
C	14.6	26.3	33.3	33.4	36.4	15.7	29.1	26.9

[a] Tablets are grouped together; tablets A and D being A. P. C. tablets and Tablets B and C, aspirin tablets, buffered and plain, respectively.

because of the wide variations noted between these tablets in the preceding run.

The data of Run B are given in Table III and Table VI. The subjects are identified as 8a to 15a inclusive, and these are different subjects from those in Run A (Table II).

Run C.—The striking difference in absorption between the two A. P. C. products (tablets A and D) noted in both Runs A and B led us to repeat the same subjects who had previously been employed in Run A. Of these seven subjects, six were available. They are referred to as 1b, 2b, etc., and are identical subjects to 1a, 2a, etc. Samples were taken at all five time intervals (six including "zero time") and determinations made in regard to salicylate and acetophenetidin absorption after administration of the A. P. C. products (tablets A and D). Tablets from the same lots which had been employed in Runs A and B were used, however they were now several months old. The data are given in Tables IV and VII.

COMMENTS UPON THE RESULTS

At the one minute sampling time, approximately 50% of the subjects on tablets A, B, and C exhibited determinable amounts of plasma salicylate, whereas only 10% of the subjects upon tablet D, responded. The surprising variation in plasma salicylate at the one minute sampling time was so extensive that it appeared not to be solely related to the acetylsalicylate content of the tablet. This massive variation between and within individuals is illustrated by the data in Tables II–VII and X. However, the possibility of this variation being partly influenced by the dosage must be considered. A dose of two of either tablet B or C provided 10 grains of acetylsalicylic acid, whereas the dosage of tablet A, provided 8 grains, and of tablet D, 7

TABLE III.—RUN B, TOTAL PLASMA SALICYLATE, μg./ml.

Tablet[a]	Subject 8a	9a	10a	11a	12a	13a	14a	15a	Average
One Minute									
A	0.6	0.0	1.0	0.0	0.0	7.0	0.5	0.4	1.2
D	0.0	0.0	0.0	0.0	0.0	2.7	0.0	0.8	0.4
B	0.0	0.0	0.4	0.0	0.3	1.1	1.3	0.8	0.5
C	1.5	0.0	0.5	0.0	0.0	0.0	0.8	0.0	0.4
Eight Minutes									
A	5.5	5.3	15.4	5.2	10.0	8.3	2.2	1.4	6.7
D	21.0	9.0	0.5	1.3	3.0	3.0	2.8	10.6	6.4
Twenty-five Minutes									
A	14.0	14.2	22.0	18.3	16.3	9.5	7.7	32.2	16.8
D	28.2	14.2	4.0	2.2	4.5	3.4	5.6	20.8	10.4
One Hour									
A	19.0	12.7	24.8	22.2	19.0	25.0	18.3	39.2	22.5
D	34.3	20.0	21.7	8.6	9.7	8.8	18.0	24.5	18.2
Two Hours									
A	23.7	10.8	21.7	25.4	23.3	26.4	28.3	35.7	24.4
D	28.3	25.3	21.3	17.9	9.7	4.6	23.8	27.6	19.8
B	37.3	25.0	31.4	20.0	25.8	21.3	40.5	42.9	30.5
C	34.2	24.0	8.3	33.7	34.0	31.0	41.4	40.3	30.9

[a] Tablets are grouped together; tablets A and D being A. P. C. tablets and Tablets B and C, aspirin tablets, buffered and plain, respectively.

TABLE IV.—RUN C, TOTAL PLASMA SALICYLATE, μg./ml.

Tablet[a]	Subject 1b	2b	3b	4b	5b	6b	7b	Average
One Minute								
A	0.0	...	0.0	0.0	0.0	0.0	0.0	0.0
D	0.0	...	0.0	0.0	0.0	0.0	0.0	0.0
Eight Minutes								
A	2.3	...	1.0	11.2	4.6	12.0	0.0	5.2
D	0.0	...	2.4	0.0	0.8	7.4	0.6	1.9
Twenty-five Minutes								
A	7.4	...	6.7	33.2	15.1	18.3	0.0	13.5
D	0.0	...	8.0	0.0	5.4	11.2	4.2	4.8
One Hour								
A	22.2	...	16.5	34.0	26.0	19.3	13.5	21.9
D	14.6	...	19.1	2.3	7.5	28.4	4.7	12.8
Two Hours								
A	25.5	...	21.8	24.7	30.6	21.8	21.0	24.2
D	18.8	...	14.0	3.3	8.2	11.8	7.6	10.6

[a] Tablets A and D are A. P. C. tablets.

grains. Notwithstanding this, the administration of lesser amounts of acetylsalicylic acid in tablet A, resulted in an equal or higher concentration of plasma salicylate.

Combining all the data observed at the one minute sampling time results in an approximately equal average plasma salicylate concentration following the use of each of the acetylsalicylic acid tablets.

TABLE V.—RUN A, *p*-AMINOPHENOL BLOOD LEVELS, μg./ml.

Tablet[a]	Subject 1a	2a	3a	4a	5a	6a	7a	Average
One Minute								
A	None found							
D	None found							
Eight Minutes								
A	0.0	0.0	0.0	0.6	0.0	0.0	0.0	0.09
D	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Twenty-five Minutes								
A	4.1	0.0	3.3	2.4	0.0	1.2	4.6	2.2
D	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
One Hour								
A	6.3	2.5	4.7	5.2	0.0	1.7	6.0	3.8
D	0.0	1.6	0.0	1.4	0.0	0.0	0.0	0.4

[a] Tablets A and D are A. P. C. tablets.

TABLE VI.—RUN B, *p*-AMINOPHENOL BLOOD LEVELS, μg./ml.

Tablet[a]	Subject 8a	9a	10a	11a	12a	13a	14a	15a	Average
One Minute									
A	None found								
D	None found								
Eight Minutes									
A	1.7	1.1	0.8	0.8	1.0	1.1	0.0	1.2	1.0
D	1.3	0.0	0.0	0.0	1.6	0.0	0.0	1.2	0.5
Twenty-five Minutes									
A	2.2	2.1	1.1	0.8	1.4	1.2	1.2	3.2	1.7
D	2.3	0.0	1.2	1.2	1.8	0.0	0.0	3.5	1.3
One Hour									
A	2.9	3.2	1.4	1.8	1.8	1.8	1.5	4.0	2.3
D	3.1	0.0	3.1	1.2	3.0	3.0	1.4	4.2	2.4
Two Hours									
A	3.7	2.8	1.9	2.0	2.4	3.6	2.4	4.1	2.9
D	2.4	0.0	4.0	3.0	4.0	4.1	2.8	3.9	3.0

[a] Tablets A and D are A. P. C. Tablets.

TABLE VII.—RUN C, *p*-AMINOPHENOL BLOOD LEVELS, μg./ml.

Tablet[a]	Subject 1b	2b	3b	4b	5b	6b	7b	Average
One Minute								
A	None found							
D	None found							
Eight Minutes								
A	1.1	...	0.5	1.0	1.1	1.3	0.0	0.8
D	0.0	...	0.0	0.0	0.0	1.1	0.0	0.2
Twenty-five Minutes								
A	1.3	...	0.6	2.4	1.3	1.5	0.0	1.2
D	0.0	...	0.0	0.0	0.5	1.3	0.5	0.4
One Hour								
A	2.2	...	1.6	3.3	2.3	2.0	2.6	2.3
D	2.0	...	1.3	0.0	0.8	1.4	1.4	1.2
Two Hours								
A	2.6	...	3.4	2.0	3.2	2.5	4.0	3.0
D	2.7	...	1.4	1.0	1.0	1.3	2.2	1.6

[a] Tablets A and D are A. P. C. tablets.

However, following the use of one of the A. P. C. tablets, a considerably smaller concentration is obtained, while use of the other A. P. C. tablet results in a markedly greater concentration. This anomaly exists notwithstanding the fact that both A. P. C. tablets contain less acetylsalicylic acid than do either the plain or buffered aspirin tablets. One might presume that a marked difference in disintegration time of the tablets would account for this variation. However, when tested by the U. S. P. procedure, all tablets disintegrated in less than one minute. At the eight minute sampling time, only an occasional subject failed to exhibit a plasma salicylate level. At the twenty-five minute sampling and thereafter, the spread of plasma salicylate levels between tablets A, B, and C was not great, though that resulting from tablet A was somewhat lower. Tablet D differed markedly at all time intervals in regard to plasma salicylate levels, which were lower, and it contained the least acetylsalicylic acid. But it appears unlikely that this lesser amount of acetylsalicylic acid entirely accounts for the lower plasma salicylate levels at all time intervals, as the blood *p*-aminophenol level was also much lower, though the acetophenetidin content of tablet D was greater than tablet A. The average total conjugated *p*-aminophenol blood levels following the ingestion of two A. P. C. tablets (tablets A or D) are presented in Table IX. Here, it will be noted that at the one minute sampling time, *p*-aminophenol was not detected in any subject. In fact, out of the 42 times that the A. P. C. tablets were administered, it was found only 17 times at the eight minute sampling period, and of these, 13, or 62%, were due to the administration of tablet A, although this tablet had a lower content of acetophenetidin.

One can readily note the tremendous variations that exist between individuals, and within the same individual, following administration of the same product. This spread exists both in regard to acetylsalicylic acid and acetophenetidin, as illustrated in Table X.

TABLE VIII.—AVERAGE PLASMA SALICYLATE LEVELS, μg./ml.

Time, Minutes	Tablet A A. P. C.	Tablet D A. P. C.	Tablet B Buffered Aspirin	Tablet C Plain Aspirin
1	0.62	0.16	0.36	0.39
8	4.9	3.4	5.7	3.8
25	14.1	6.6	17.4	15.4
60	22.4	14.4	25.5	27.0
120	25.0	15.9	30.5	30.9

N = 21 for all time intervals for tablets A and D (Runs A, B, and C), except 120 minutes for which N = 14 (Runs B and C only).
N = 21 for tablets B and C at one minute (Runs A, B, and C).
N = 7 at 8, 25 and 60 minutes (Run A only), and N = 8 at 120 minutes (Run B only).

TABLE IX.—AVERAGE TOTAL CONJUGATED *p*-AMINOPHENOL LEVEL IN WHOLE BLOOD, μg./ml.

Time, Minutes	Tablets A A. P. C.	Tablets D A. P. C.
1	0.0	0.0
8	0.6	0.3
25	1.7	0.6
60	2.8	1.4
120	2.9	2.2

N = 21 (Runs A, B, and C) at all time intervals except 120 minutes for which N = 14 (Runs B and C).

TABLE X.—REPRESENTATIVE DATA EXEMPLIFYING VARIATION BETWEEN AND WITHIN INDIVIDUALS AFTER ADMINISTRATION OF TABLET A (A. P. C.)

	Time, Minutes 1	8	25	60
Plasma Salicylate Level, μg./ml.				
Subject 4				
Run A	0.3	2.0	5.8	20.6
Run C	0	11.2	33.2	34.0
Subject 6				
Run A	1.4	2.2	8.3	13.9
Run C	0	12.0	18.3	19.3
Blood *p*-Aminophenol Level, μg./ml.				
Subject 1				
Run A	0	0	4.1	6.3
Run C	0	1.1	1.3	2.2
Subject 7				
Run A	0	0	4.6	6.0
Run C	0	0	0	2.6

STATISTICAL TREATMENT OF DATA

In order to utilize all of the data, evaluation was made separately at each time level using Wilcoxon's rank test for paired variates (11, 12). This method is nonparametric (i. e., free of assumption about normality and equality of variances). All three runs were included as though three different groups of individuals were used. The summations for plasma salicylate and blood *p*-aminophenol are given in Tables XI and XII, respectively.

In these tables N = number of nonzero differences, P = probability, and R = lower rank sum of differences.

A positive R indicates that the first tablet in a comparison gives higher levels. A negative R indicates that the second tablet in a comparison gives higher levels. A zero R indicates that all the differences were either positive or negative.

Upon this statistical treatment of the data, the following conclusions are drawn.

(*a*) Plasma salicylate is statistically significantly higher (P = 0.01–0.02) following tablet A (A. P. C.) than tablet D (A. P. C.) at all time intervals except at eight minutes. Blood *p*-aminophenol is statistically significantly higher following tablet A (P = 0.01–0.02) at all time intervals except two hours.

The fact that tablet A contains 14% more salicylate appears to be no more influencing than the fact that tablet D contains 20% more acetophenetidin.

(*b*) The average plasma salicylate following tablet A (A. P. C.) is higher at one minute than that following table B (buffered aspirin), or table C (plain aspirin), but this difference is not statistically significant. At all other time intervals, the plasma salicylate was higher for both buffered aspirin and plain aspirin, but again not at a statistically significant level. One should again mention that the aspirin tablets contained 25% more acetylsalicylic acid, yet failed to show significant difference at this differential dosage as compared to tablet A.

TABLE XI.—STATISTICAL TREATMENT OF PLASMA SALICYLATE LEVELS (WILCOXON'S RANK TEST FOR PAIRED VARIATES), TABLET *vs.* TABLET

Time Comparisons		A *vs.* D	A *vs.* B	A *vs.* C	B *vs.* D	C *vs.* D	B *vs.* C
1 minute	N	10	11	11	7	9	11
	R	+4	+22	+25	+7	+14	−32
	P	0.01–0.02	0.3	0.5	0.2	0.3	0.9
8 minutes	N	21	7	7	6	7	7
	R	+72	−4.5	−7	0	+3	+11
	P	0.1	0.1	0.2	0.02	0.06	0.6
25 minutes	N	20	7	7	7	7	7
	R	+24	−6	−5	0	0	−14
	P	0.01	0.2	0.1	0.01	0.01	1.0
1 hour	N	21	7	7	7	6	7
	R	+37	−11	−10	+1	+1	+15
	P	0.01	0.6	0.5	0.02–0.05	0.02–0.05	0.9
2 hours	N	14	8	8	8	8	8
	R	+14	−5	−8	+1	+5	−17
	P	0.01–0.02	0.06	0.2	0.01–0.02	0.06	0.9

(*c*) The plasma salicylate level following the administration of two plain aspirin or buffered aspirin tablets (tablets B and C) is not statistically significantly different at any of the sampling times, for this group of subjects.

GENERAL DISCUSSION

The results indicate that plasma salicylate levels following the oral ingestion of aspirin or aspirin containing compounds are not solely dependent upon the dosage of aspirin administered, providing the dose is reasonably approximate. In this study, and among these tablets, a tablet containing 4 grains of aspirin (8 grains per dose) appears to be absorbed almost to the same extent as the aspirin in tablets which contain 5 grains (10 grains per dose). However, a tablet which contains $3^1/_2$ grains of aspirin (7 grains per dose) produces a far lower plasma salicylate level than any of the other tablets. That this lower plasma concentration of salicylate following use of the $3^1/_2$ grain tablet is not solely due to the lower acetylsalicylate intake is suggested by the results obtained as regards acetophenetidin absorption. Furthermore, it is interesting to note that not in all cases could salicylate be demonstrated in the plasma at "one minute." The information obtained with respect to "one minute absorption" of salicylates following oral administration to the same and to different individuals on different days, indicates that absorption at this early sampling time is quite unpredictable between or within individual subjects. Inverse conclusions are to be drawn from the comparison of the absorption of acetophenetidin following oral administration of tablet A and tablet D. Although tablet D contains 3 grains acetophenetidin and tablet A, $2^1/_2$ grains acetophenetidin per tablet, the blood levels resulting from ingestion of tablet A are significantly higher than those produced by tablet D at each of the time intervals where it was detected (Tables VIII and IX).

Since plasma salicylate levels and blood total conjugated *p*-aminophenol levels do not appear to be solely related to the dosage of aspirin or acetophenetidin, we may speculate that the factors which may play a role in the absorption of orally ingested analgesics in tablet form are: (*a*) The disintegration properties of a tablet which may be influenced by the kind and quantity of the disintegrator, the kind and quantity of the lubricant, whether the granulation is prepared by the wet or dry process, and its size and, finally, by the degree of compression and the amount of heat generated during compression; (*b*) the emptying time of the stomach; (*c*) the pH of the gastric contents; (*d*) the amount of mucous in the stomach; and (*e*) the amount of food or chyme in the stomach.

TABLE XII.—STATISTICAL TREATMENT OF BLOOD *p*-AMINOPHENOL LEVELS (WILCOXON'S RANK TEST FOR PAIRED VARIATES) TABLET A *vs.* D

Time →	8 Min.	25 Min.	1 Hr.	2 Hr.
N	12	19	20	14
R	−4.5	−25	−39.5	−34.5
P	0.01	0.01	0.01–0.02	0.3

Although *in vitro* tablet disintegration time may be constant for a given product, it may vary tremendously between products, and this variation may be a highly significant factor, for it could permit the tablet to become coated and "blocked" by mucous. Certainly *in vitro* disintegration time studies are most useful in providing a reasonable means of manufacturing control, but we are of the opinion that tablets with the same *in vitro* disintegration times, do not necessarily possess the same *in vivo* disintegration times. The other factors enumerated which are all physiologic will not only vary between individuals, but may vary within the same individual from day to day, or even within a shorter time period, and thus drastically alter day to day data upon absorption of any similarly administered medicament.

CONCLUSIONS

The following conclusions are offered as being apropos.

1. Plasma salicylate levels or blood *p*-aminophenol levels following the oral ingestion of aspirin or aspirin-acetophenetidin containing products are not necessarily solely and directly related to the aspirin or acetophenetidin content

of the tablets, when this content is reasonably close.

2. The degree of salicylate or of acetophenetidin absorption is of a wide variation within the same individuals for the same product upon different days, and is probably governed by several physiologic factors as well as the composition and physical characteristics of the tablets.

3. It is possible to demonstrate salicylate in the plasma within one to two minutes after ingestion of tablets containing aspirin at a dose level of seven to ten grains.

4. *p*-Aminophenol is not present in the blood within one to two minutes, but does occur in about fifty per cent of the subjects within eight minutes.

5. The data observed are treated statistically to aid in evaluating the widely spread individual figures.

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Analogs of Nucleotides III*

Syntheses in the Series of Adenosine Phosphonate Derivatives

By MANFRED E. WOLFF† and ALFRED BURGER

The synthesis of 9-[5′-deoxy-5′-(diethyl phosphonate-β-D-ribofuranosyl)]-6-amino-2-(methylthio)purine is described. Methyl 2,3-isopropylidene-5-deoxy-5-(diethyl phosphonate)-D-ribofuranoside was converted to the aldehydo triacetate in several steps. Treatment with 2-(methylthio)-4,6-diaminopyrimidine gave a Schiff base which was transformed to the N-ribofuranoside, coupled with 2,5-dichlorophenyldiazonium sulfate and reduced. Thioformylation and cyclization to the purine gave the final product.

THE SIGNIFICANCE of the C-O-P moiety in adenosine phosphate with respect to the various reactions in which this metabolite is involved should be capable of examination by biochemical evaluation of adenosinephosphonic acid and the esters and anhydrides derived from it. The C-P bond in such phosphonic acid analogs would not be subject to hydrolysis, and its degradation, if any, would occur at a rate of much smaller magnitude than is found in adenosine phosphate. The phosphonic acid analogs are, therefore, potential adenosine phosphate antagonists since they could block essential cellular receptor sites by virtue of their pronounced structural similarity to the natural phosphate factors.

The preparation of phosphonoglucopyranosyl derivatives of adenine as model compounds for syntheses involving phosphonoribofuranosyl groups has been described by us in an earlier study (1). This paper is a progress report on experiments designed to furnish ultimately adenosine-5′-phosphonic acid.

The synthetic route employed for the preparation of the glucopyranosyl derivatives involved the treatment of a 6-acylamido-9-(2′,3′,4′-tri-O-acetyl-6′-deoxy-6′-bromo-β-D-glucopyranosyl) purine with a trialkyl phosphite in a Michaelis-Arbuzov transformation. By analogy, the most obvious preparation of an adenosine-5′-phosphonic acid derivative would involve the similar utilization of a 5′-deoxy-5′-haloadenosine derivative. These halonucleosides do not exist as such, however, but in the form of cyclonucleoside quaternary salts (2). It was necessary, therefore, to use a synthetic scheme in which a suitably protected 5-ribosephosphonate fragment could be introduced into a second moiety which could then be converted into an adenine structure.

Among the methods which satisfy these requirements are (*a*) the glycosidation of an imid-

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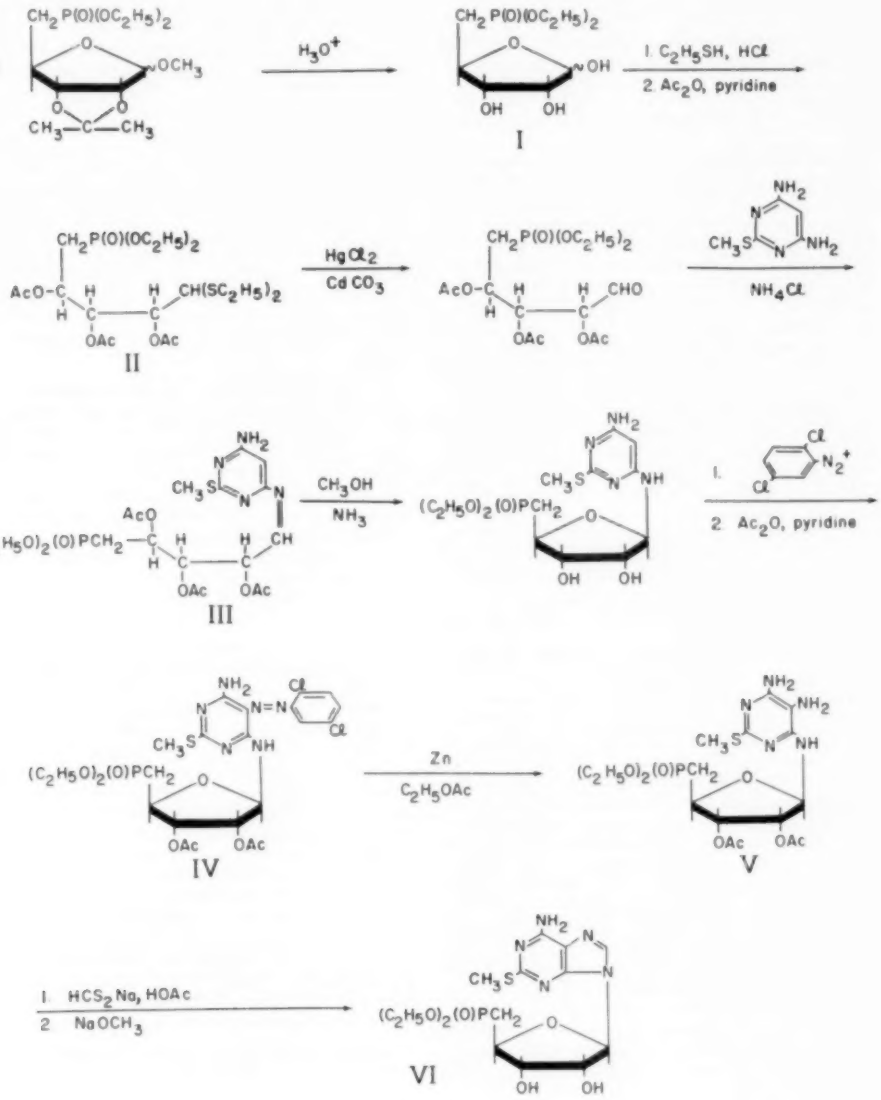
azole derivative followed by cyclization to a purine (3), or (*b*) the ribosidation of a pyrimidine derivative followed by cyclization to a nucleoside (4). The latter method was selected in the present synthesis.

D-Ribose was converted in four steps to methyl 2,3-isopropylidene-5-deoxy-5-(diethyl phosphonate)-D-ribofuranoside (1). The masking groups were removed by acid hydrolysis and the 5-deoxy-5-(diethyl phosphonate)-D-ribofuranose (I) was characterized as its osazone. Treatment with ethanethiol in hydrochloric acid medium and subsequent acetylation gave 2,3,4-tri-O-acetyl-5-deoxy-5-(diethyl phosphonate)-D-ribose diethyl mercaptal (II) which was purified by molecular distillation or chromatography on alumina. Removal of the mercaptal groups with mercuric chloride regenerated the aldehyde function, and the product was condensed with 2-(methylthio)-4,6-diaminopyrimidine to give the corresponding Schiff base (III).

For the introduction of the 5-amino function into the pyrimidine, coupling with 2,5-dichlorophenyldiazonium sulfate according to Todd's



method (5) was chosen in order to avoid destruction of the ribosidic linkage. The Schiff base (III) was deacetylated with methanolic ammonia, coupled, and the resulting azo dye was purified by adsorption on chromatographic alumina and elution through conversion to the acetylated derivative (IV). Zinc dust reduction cleaved the azo group to produce 5,6-diamino-4-(2′,3′-di-O-acetyl-5′-deoxy-5′-(diethyl phosphonate)-D-ribofuranosylamino) - 2 - (methylthio)pyrimidine (V). Although this compound was not obtained in a state of complete analytical purity, it was thioformylated with dithioformic acid, deacylated, and cyclized to furnish 9-[5′-deoxy-5′-(diethyl phosphonate)-β-D-ribofuranosyl]-6-amino-2-(methylthio)purine (VI) which was characterized as the chloroplatinate.

EXPERIMENTAL

5-Deoxy-5-(diethyl phosphonate)-D-ribose.—A stirred suspension of 33.2 Gm. (0.102 mole) of syrupy methyl 2,3-isopropylidene-5-deoxy-5-(diethyl phosphonate)-D-ribofuranoside (1) in 300 cc. of 0.4 *N* sulfuric acid was heated on the steam bath for five hours. The resulting orange-brown solution was filtered from a small amount of insoluble oil, neutralized to pH 7 with barium carbonate, filtered, and partially decolorized with Darco. The filtered solution was evaporated at 50° to give 24.8 Gm. (90%) of a thick dark syrup which did not crystallize.

A sample (500 mg.) was dissolved in 4 cc. of water, 1.2 Gm. of phenylhydrazine in 6 cc. of 50% acetic acid was added, and the solution was heated on the steam bath (6). The solution set to a solid mass after five minutes, 30% acetic acid was added to cover the crystals, and heating was continued for forty minutes. The canary-yellow osazone was isolated by filtration, washing with 30% acetic acid and water, and recrystallization from absolute alcohol, m. p. 194–196° (decompn.).

Anal.—Calcd. for $C_{21}H_{29}N_4O_5P$: C, 56.24; H, 6.52. Found: C, 55.87; H, 6.70.

2,3,4-Tri-O-acetyl-5-deoxy-5-(diethyl phosphonate)-D-ribose diethyl mercaptal.—A stirred, ice-cold solution of 55.5 Gm. (0.205 mole) of 5-deoxy-5-(diethyl phosphonate)-D-ribose in 75 cc. of concentrated hydrochloric acid was treated with 100 Gm. of ethyl mercaptan in 4 portions. The mixture was then stirred at 0° for ten minutes, at 27° for fifteen minutes, and at 40° for ten minutes. It was poured carefully into 800 cc. of saturated sodium bicarbonate solution with stirring, and the pH adjusted to 7 with solid sodium bicarbonate. The filtered solution was extracted with 1.5 L. of chloroform in divided portions, and the chloroform washed with 40 cc. of water. The dried, filtered solution was evaporated to a dark oil *in vacuo*. The oil was dissolved in 300 cc. of dry pyridine, treated with 75 cc. of acetic anhydride, and stored at 27° for sixteen hours. The solution was cooled in ice, excess acetic anhydride was decomposed by careful addition of 40 cc. of water, and 800 cc. of chloroform was added. Then 20% sulfuric acid was added in the cold until pH 2, and the layers were separated. The acid layer was extracted with 600 cc. of chloroform in divided portions and the combined chloroform extracts were washed with saturated sodium bicarbonate solution until basic, with water until neutral, and dried with sodium sulfate. Evaporation of the chloroform *in vacuo* gave a syrup (47 Gm.) which was chromatographed on an alumina column (2.2 x 37 cm.) in chloroform to give 33.5 Gm. (34%) of a yellow syrupy product. A sample was purified by molecular distillation at 0.1 mm., bath temperature 170°.

Anal.—Calcd. for $C_{19}H_{35}O_8PS_2$: C, 46.89; H, 7.25. Found: C, 47.41; H, 7.94.

Aldehydo-2,3,4-tri-O-acetyl-5-deoxy-5-(diethyl phosphonate)-D-ribose.—To a stirred solution of 23.0 Gm. (0.0473 mole) of 2,3,4-tri-O-acetyl-5-deoxy-5-(diethyl phosphonate)-D-ribose diethyl mercaptal and 44.5 Gm. of cadmium carbonate in 85 cc. of acetone and 30 cc. of water there was added, dropwise, a solution of 46.2 Gm. (0.17 mole) of mercuric chloride in 86 cc. of acetone during three hours. The mixture was stirred for 15 hours, and 5 Gm. of cadmium carbonate and 15 cc. of water was added. After two hours of further stirring, the mixture was refluxed for one hour, the solids were filtered, and the filtrate was evaporated and azeotroped with benzene. The residue was extracted with 300 cc. of chloroform in divided portions and the filtered chloroform extracts were washed with 100 cc. of 40% potassium iodide solution and water. The dried, filtered solution was evaporated to leave 14.9 Gm. of dark syrup which could not be crystallized or distilled.

2-(Methylthio)-4,6-diaminopyrimidine.—The product has been described (7) but the preparation was modified. To a solution of 7.55 Gm. (0.05 mole) of 2-thio-4,6-diaminopyrimidine hemihydrate (8) in 50 cc. of *N* sodium hydroxide and 25 cc. of water was added, slowly with shaking, 7.1 Gm. (0.05 mole) of methyl iodide. After one hour, the mixture was acidified to pH 5 with acetic acid and the product removed. It was recrystallized from hot water (Darco) and formed 4.95 Gm. (63%) of colorless needles, m. p. 185–186°. The reported (7) melting point is 185–186°.

6-Amino-4-[2′,3′,4′-tri-O-acetyl-5′-deoxy-5′-(diethyl phosphonate)-D-ribosamino]-2-(methylthio)-pyrimidine.—A solution of 17.5 Gm. (0.111 mole) of 2-(methylthio)-4,6-diaminopyrimidine, 10.5 Gm. (0.028 mole) of aldehydo-2,3,4-tri-O-acetyl-5-deoxy-5-(diethyl phosphonate)-D-ribose and 0.4 Gm. of ammonium chloride in 360 cc. of boiling absolute ethanol was allowed to stand at 28° for eighteen hours. The solvent was removed *in vacuo*, and the product dissolved in 50 cc. of chloroform. The filtered solution was poured onto an alumina column (1.5 x 25 cm.) and the column washed with fresh chloroform. The first 50-cc. fraction contained no product; the next 100 cc. was dark and gave a phosphorus-containing gum upon evaporation *in vacuo* weighing 7.2 Gm. Further fractions gave pure starting pyrimidine, which, when combined with the chloroform-insoluble residue weighed 12.38 Gm., so that 5.12 Gm. of the pyrimidine was consumed.

6-Amino-4-[2′,3′-di-O-acetyl-5′-deoxy-5′-(diethyl phosphonate)-D-ribofuranosylamino]-5-(2″,5″-

dichlorophenylazo) - 2 - (methylthio)pyrimidine.—A solution of 4.2 Gm. of crude 6-amino-4-[2′,3′,4′-tri-O-acetyl-5′-deoxy-5′-(diethyl phosphonate)-D-ribosamino]-2-methylthiopyrimidine in 100 cc. of methanol which had been saturated with ammonia at 0°, was allowed to stand for thirty-six hours at 25°. It was evaporated to dryness *in vacuo* and left a resin which was dissolved in 200 cc. of pyridine. The pyridine solution was poured into a stirred, ice-cold neutral diazotized solution obtained from 1.6 Gm. of 2,5-dichloroaniline. The mixture was stirred for two hours at 0°, diluted with 600 cc. of water, cooled to 0°, and the product was filtered and dried (2.0 Gm.). The orange powder was soluble in ethyl acetate and pyridine. It was dissolved in 100 cc. of pyridine and chromatographed on alumina (column 1.5 x 40 cm.). Washing the column with pyridine gave phosphorus-free azopyrimidine while the ribosidic material remained adsorbed. The column was extruded, dried, and stirred for eighteen hours with 200 cc. of pyridine and 50 cc. of acetic anhydride. The excess anhydride was destroyed by adding ethanol to the cold solution and the mixture was filtered. The alumina was washed thoroughly with hot ethyl acetate and the combined filtrate and washings were evaporated *in vacuo*. The residue, a dark glass which did not crystallize, was heated with ethanolic picric acid and gave a picrate benzenate, yellow needles, m. p. 164–165° (softening at 160°) after recrystallization from benzene. Heating the product at 58° *in vacuo* in an attempt to remove the benzene resulted in loss of picric acid.

Anal.—Calcd. for $C_{20}H_{34}Cl_2N_5O_{15}P \cdot C_6H_6$: C, 44.45; H, 4.15. Found: C, 44.54; H, 3.45.

Attempts to diazotize 2,5-dichloroaniline according to Baddiley, *et al.* (9), resulted in an insoluble diazoaminobenzene derivative, a difficulty encountered by Noelting and Kopp in earlier work (10). The procedure of these authors gave much better results: A hot, rapidly stirred solution of 1.6 Gm. of 2,5-dichloroaniline in 4 cc. of concentrated sulfuric acid and 10 cc. of water was cooled rapidly and the fine crystal mass treated very slowly with a solution of 0.72 Gm. of sodium nitrite in 15 ml. of water at 5°. The filtered yellow solution was treated with a little urea and neutralized to pH 6 with pyridine.

5,6-Diamino-4-[2′,3′-di-O-acetyl-5′-deoxy-5′-(diethyl phosphonate)-D-ribofuranosylamino]-2-(methylthio)pyrimidine.—A suspension of 120 Gm. of zinc dust and 15 Gm. of crude 6-amino-4-[2′,3′-di-O-acetyl-5′-deoxy-5′-(diethyl phosphonate)-D-ribofuranosylamino]-5-(2″,5″-dichlorophenylazo)-2-(methylthio)pyrimidine in 450 cc. of refluxing ethyl acetate was stirred rapidly while 60 cc. of glacial acetic acid in 600 cc. of ethyl acetate was added dropwise during one hour. The colorless mixture was filtered, the zinc washed three times with 200 cc. of boiling ethyl acetate, and the combined liquids were evaporated under nitrogen *in vacuo*. The residue was washed with petroleum ether and ether, and an alumina column (2.2 x 35 cm.) was prepared using ether. The petroleum ether and ether wash liquids were passed through and then the residue dissolved in 50 ml. of chloroform was poured on. The column was developed with ether, chloroform-ether 1:1, 1% ethanol in chloroform, and ethanol. The phosphorus-containing material was eluted by chloroform. A portion was isolated as the yellow picrate by treatment with ethanolic picric acid and recrystallization from benzene, m. p. 140° (decompn.) (rapid heating).

9-[5′-Deoxy-5′-(diethyl phosphonate)-β-D-ribofuranosyl]-6-amino-2-(methylthio)purine.—An ice-cold solution of 3 Gm. of chromatographed 5,6-diamino-4-[2′,3′-di-O-acetyl-5′-deoxy-5′-(diethyl phosphonate)-D-ribofuranosylamino]-2-(methylthio)pyrimidine in 100 cc. of absolute alcohol was treated with sodium dithioformate prepared by substituting anhydrous, xylene-dried sodium sulfide in the method of Levi (11). This salt (2.4 Gm.) was dissolved in 15 cc. of alcohol and to it was added 0.6 cc. of acetic acid in 15 cc. of absolute alcohol. The mixture was allowed to stand for four hours at 0°, treated with 1.2 Gm. and 0.3 cc. of the same reagents, and stored overnight at 30°. After refluxing for one hour and evaporation of the solvent *in vacuo*, the residue was dissolved in 100 cc. of boiling ethyl acetate, filtered, evaporated *in vacuo*, dissolved in 30 ml. of benzene, decanted from a small residue, and poured onto 30 Gm. of alumina (column 2.2 cm. diameter). The column was washed with benzene until the washings were colorless, and the adsorbed dark band was eluted with 100 ml. of pyridine. Evaporation of the pyridine gave 500 mg. of a solid which was dissolved in 20 cc. of absolute methanol and treated with 1.5 ml. of *N* methanolic sodium methoxide solution. It was refluxed for four hours and stored for twelve hours at 30°. It was neutralized to pH 7 with glacial acetic acid and evaporated to a crystalline residue *in vacuo*. The residue was extracted with 100 cc. of boiling chloroform and the filtered extracts were evaporated *in vacuo* to leave a small amount of brown crystalline residue, soluble in hot water, alcohol, acetone, chloroform, and benzene; and insoluble in cold water, petroleum ether, and ether. It was washed with cold ether, and gave a poorly crystalline picrate. Treatment of the residue with platinic chloride in absolute ethanol gave the chloroplatinate as an amorphous powder which was washed with cold absolute alcohol, centrifuged, and freed of solvent by decantation and drying.

Anal.—Calcd. for $C_{15}H_{24}N_5O_6PS \cdot {}^1/_2H_2PtCl_6 \cdot 2H_2O$: C, 26.71; H, 4.33. Found: C, 26.68; H, 4.12.

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Studies on the Inhalation of Vapors from Radioactive Menthol and Camphor*

By ROBERT L. BOGNER and THOMAS C. GRUBB

Tritium "tagged" camphor and *l*-menthol were combined with other essential oils in a petrolatum base. Samples of bronchial air from dogs inhaling vapors of the "tagged" ointment in a closed system showed radioactivity after the second respiration. Air collected over an open container covered with the tagged ointment showed activity for a period of twelve hours' duration. Samples of bronchial air collected after the ointment was placed on the dog's chest showed activity up to twelve hours after a single application.

THE INHALATION of vapors from aromatic oils has been employed for centuries in the treatment of respiratory diseases. In recent years, volatile substances have also been inhaled for the treatment of sinusitis, bronchitis, asthma, laryngitis, etc., McClellan (1) and Currence (2). Since there is scant information available on the speed and duration of these inhaled vapors, it was decided to use radioactive campor and menthol as "tracers" for these determinations.

EXPERIMENTAL

Camphor and *l*-menthol were separately irradiated with tritium by the method of Wilzbach (3). Specific activities of 45.5 mc. per Gm. and 62.5 mc. per Gm., respectively, were obtained. These materials were added to other volatile substances (oil of nutmeg, oil of cedarleaf, oil of eucalyptus, and thymol) in a petrolatum base, thus providing a specific activity in the final mixture of 4.3 mc. per 10 Gm. of ointment.

The dogs used in these experiments were prepared as follows: Under intravenous sodium pentobarbital anesthesia, the left chest wall was entered through the fourth intercostal space and a polyethylene tube was inserted into an opening made in the bronchus just below the bifurcation in two animals, and just below the second main division of the bronchus in three other dogs. The tubes were sewed into position so that the samples of bronchial air from each respiration could be collected outside of the chest wall.

In a typical experiment, 10 Gm. of the radioactive ointment were spread over an area (8 cm. x 10 cm.) inside of a four-liter glass bottle which was partially submerged in a water bath maintained at $35 \pm 0.5°$ (skin temperature). The dog was placed under pentobarbital anesthesia and its nose positioned into a mask connected to the bottle containing the tritiated ointment. Suitable valves and tubing were provided so that at each inhalation, air was drawn over the ointment and into the dog's respiratory tract, as shown in Fig. 1. Samples of expired air were collected by placing the tubing from the dog's bronchus inside a small glass bottle filled with water and submerged in a shallow tray of water. About 5 cc. of air were collected at each expiration. Separate sample bottles were filled with each expiration. The bottles were capped under water and taken to the counting laboratory, where the radioactivity of their contents was determined as follows: A two-liter counting chamber was evacuated to about 10 μ and the sample allowed to expand into the counting chamber, which was then filled to atmospheric pressure with methane. The counting chamber was placed inside a ring of anticoincidence counters surrounded by a steel shield, 20.5 cm. thick. The sample was then counted on the plateau in the proportional counting region. The normal background count was 60 c. p. m. (counts per minute). Samples of the expired air were collected after the 2nd, 4, 5, 6, 7, 10, 13, 15, and 19th respirations following the initial inhalation of the vapor-containing air. Thus, air samples were obtained as early as four seconds and as late as eighty seconds after the initial inspiration of the "tagged" vapors.

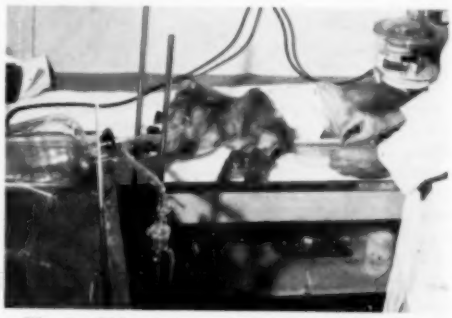

Fig. 1—This photograph shows the glass bottle containing the "tagged" ointment immersed in a water bath. The vapors are inhaled through the mask over the dog's nose and the inhaled air is collected from the tube coming from the dog's chest. The investigator is collecting the air samples under water.

The results indicated that irradiated vapors were detectable after the second respiration (five and eight seconds) in two of the dogs. Samples from the other dogs contained "tagged" vapors after the 5th, 7th, and 10th respirations. The specific activity of these samples ranged from 100 net c. p. m. per cc. of air after five seconds contact to 1,000 c. p. m. per cc. of air in the sample taken thirty-three seconds after the initial inhalation.

In order to determine the duration of therapeutic vapors released from the ointment application area, an *in vitro* and an *in vivo* experiment were carried out as follows:

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Fig. 2.—This photograph indicates the position of the dog's nose in relation to the "tagged" ointment rubbed on his chest. Note the indwelling tube, coming from the intercostal space, to which a syringe is attached to collect the air sample.

In the *in vitro* experiment, 10 Gm. of the "tagged" ointment having an activity of 11 mc. per Gm., were spread over the bottom of a stainless steel pan (15 x 25 x 5 cm.) resting in a water bath maintained at 35 ± 0.5°. A glass funnel 5 cm. in diameter was inverted 2.5 cm above the surface of the ointment. At various time intervals, 25 cc. of air were drawn into a glass syringe connected to the stem of the funnel by a 5-cm. length of Tygon tubing. These samples were assayed for tritium activity, according to the method described. Samples of air were taken after 1 minute, 30 minutes, and one, three, five, eight, and twelve hours, and the activity reported for each of these samples, respectively, was as follows: 3,880, 1,393, 750, 120, 93, 177, and 90 net c. p. m. per cc. of air.

In the *in vivo* experiment, a dog prepared with an indwelling bronchial cannula just below the bifurcation, as described, was treated as follows: Following anesthesia of the animal with sodium pantobarbitəl, 10 Gm. of the radioactive ointment were spread over an area 7.5 x 7.5 cm. on the shaved chest and covered with a light piece of gauze. The dog's nose was positioned 7.5 cm. from the inuncted area as shown in Fig. 2. Samples of air were withdrawn by aspiration from the cannula into a 30-cc. glass syringe at the rate of 5 cc. per respiration until a 25-cc. sample was collected in the syringe. This experiment was conducted out-of-doors with a temperature of 21–27°, and the samples collected after eight and twelve hours' exposure to the vapors were found to have a specific activity of 958 and 647 net c. p. m. per cc. of air, respectively.

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Catalytic Hydrogenation of Derivatives of α-Cyano-β-(1-naphthyl)acrylic Acid*

By JAMES E. GEARIEN, MASUMI NAKAMICHI, and KENNETH J. LISKA†

The ethyl ester and amide of α-aminomethyl-β-(1-naphthyl)propionic acid were prepared by the catalytic hydrogenation of the corresponding cyanonaphthylacrylic acid derivative. These compounds may be regarded as analogs of lysergic acid derivatives.

DERIVATIVES of α aminomethyl-β-(1-naphthyl) propionic acid (II) possess molecular structures that might be considered analogous to rings B, C, and D of the lysergic acid molecule (III) and, therefore, might possess the pharmacological activity of the ergot alkaloids. The amide and ethyl ester of α-aminomethyl-β-(1-naphthyl)-propionic acid were prepared by the catalytic hydrogenation of the amide and ethyl ester of α-cyano-β-(1-naphthyl)acrylic acid (I).

Ethyl α-cyano-β-(1-naphthyl)acrylate has been made by the condensation of the sodium salt of cyanoacetic acid with 1-naphthaldehyde to yield the sodium salt of α-cyano-β-(1-naphthyl)-acrylic acid which was then esterified (1). A single step synthesis, reported by Fulton and Robinson (2), utilized the condensation of ethyl cyanoacetate with 1-naphthaldehyde. By using morpholine as the catalyst instead of the recommended piperidine and by removing the water as it is formed during the reaction, the yield of ethyl α-cyano-β-(1-naphthyl)acrylate was increased from 48 to 75 per cent of the theoretical.

The previously unreported α-cyano-β-(1-naphthyl)acrylamide was synthesized by the condensation of cyanoacetamide with 1-naphthaldehyde. Maximum yields were obtained when morpholine was used as the catalyst and when the reaction was carried out without a solvent.

Hydrogenations of the ester and amide of α-cyano-β-(1-naphthyl)acrylic acid were carried out at room temperature and low pressures using W-1 Raney nickel as the catalyst. These

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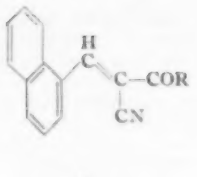
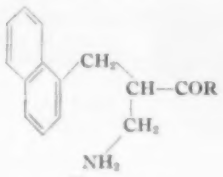
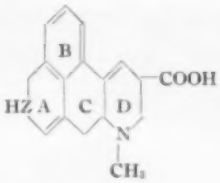


I
R=NH_2, OC_2H_5



II
R=NH_2, OC_2H_5



III

reactions were successful only when a large ratio of catalyst to hydrogen acceptor was employed and when ammonia was added to the hydrogenation mixture to reduce the formation of secondary amines (3, 4). Successful hydrogenation of α-cyano-β-(1-naphthyl)acrylamide was possible only when the weight ratio of catalyst to hydrogen acceptor was as great as two to one. Under these conditions and in the presence of small amounts of ammonia, crude yields were as high as 77 per cent of the theoretical, but the removal of impurities resulted in the loss of considerable amounts of the product. Hydrogenation of ethyl α-cyano-β-(1-naphthyl)acrylate proceeded smoothly with a one to one ratio of catalyst to hydrogen acceptor to give a 51 per cent yield of the expected product. Without the addition of ammonia, the yield was only half of that reported. The amount of ammonia, however, could not be further increased without reducing the yield of desired product due to ammonolysis.

The ultraviolet absorption curves of the two α-aminomethyl-β-(1-naphthyl)propionic acid derivatives in 95 per cent ethanol exhibited three maxima in the region 270–295 mμ, indicative of an intact naphthalene system with no exocyclic conjugation (5).

In a preliminary pharmacological screening, the compounds did not exhibit pharmacological activity.[1]

EXPERIMENTAL[2]

Ethyl α-Cyano-β-(1-naphthyl)acrylate.—A mixture of 15.6 Gm. (0.1 mole) 1-naphthaldehyde, 11.3 Gm. (0.1 mole) ethyl cyanoacetate, one ml. of morpholine, and 75 ml. of dry benzene was heated at reflux temperature in a Dean-Stark moisture determination apparatus until the water collected came to a constant volume. After the benzene was removed by distillation under reduced pressure, the residual oil solidified on cooling. The solid was recrystallized from 80 ml. of boiling ethanol using charcoal for decolorization. This gave 18.5 Gm. (74%) of yellow needles, m. p. 81.5–82°. Hopkins and Chisholm (1) reported 81–82°.

α-Cyano-β-(1-naphthyl)acrylamide.—A mixture of 15.6 Gm. (0.1 mole) of 1-naphthaldehyde and 8.4 Gm. (0.1 mole) of cyanoacetamide was heated on the steam bath for five minutes. Morpholine (1 ml.) was quickly added, the reaction flask was fitted with a reflux condenser and agitated briskly. Heating was resumed and in a few minutes the contents of the flask solidified. After an additional ten minutes of heating, the flask was cooled to room temperature, its contents ground up in a mortar, and the solids treated with 20 ml. of ethanol to remove most of the color. One recrystallization from a large volume of ethanol then gave 16.6 Gm. (74%) of cream-colored needles, m. p. 194–195°. After a second recrystallization from absolute ethanol-acetone (1:1), the analytically pure sample melted at 195.5–196°λ max. (95% ethanol): 257, 330 (broad) mμ.

Anal.—Calcd. for $C_{14}H_{10}N_2O$: N, 12.61. Found: N, 12.66.

Ethyl α-Aminomethyl-β-(1-naphthyl)propionate.—A 10.1-Gm. (0.04 mole) sample of finely powdered ethyl α-cyano-β-(1-naphthyl)acrylate was suspended in 210 ml. of absolute ethanol. To this suspension was added 11.6 Gm. of freshly prepared W-1 Raney nickel (6) and 10.4 ml. of ethanolic ammonia solution containing 1.4 Gm. (0.08 mole) of ammonia. The mixture was hydrogenated for three and one-half hours at an initial pressure of 46 p. s. i. The contents of the pressure bottle were filtered free of catalyst, and the filtrate evaporated. A light yellow oil remained. It was distilled under reduced pressure, b. p. 132–138° (0.11 mm.). The yield of pale yellow oil was 5.2 Gm. (51%). λ max. (95% ethanol): 272.5, 282.5, 291 mμ.

Anal.—Calcd. for $C_{16}H_{19}NO_2$: C, 74.68; H, 7.44; N, 5.44. Found: C, 74.77; H, 7.46; N, 5.25.

α-Aminomethyl-β-(1-naphthyl)propionamide.—To a suspension of α-cyano-β-(1-naphthyl)acrylamide (4.4 Gm., 0.02 mole) in 220 ml. of absolute ethanol was added 8.0 Gm. of W-1 Raney nickel and 18.5 ml. of an ethanolic ammonia solution which contained 3.4 Gm. (0.02 mole) of ammonia. The reactants were shaken under an initial hydrogen pressure of 46 p. s. i. for four hours. After the catalyst was removed, the solvent was evaporated, and the residue, which had solidified to a hard mass when cooled to room temperature, was broken up and triturated with 15 ml. of anhydrous ether. A tan powder resulted (3.52 Gm., crude yield) which was recrystallized from a small volume of chloroform to give 1.9 Gm. (42%) of white crystals, m. p. 157–158°. For analysis, the compound was further purified by suspending the 1.9 Gm. in 25 ml. of water, adding 25 ml. of concentrated hydrochloric

[1] The authors are indebted to Abbott Laboratories for subjecting the compounds to their pharmacological screening procedures.

[2] Melting points are not corrected. Analyses were performed by Weiler and Strauss Analytical Laboratories, Oxford, England.

acid, filtering the solution, and then making alkaline with 20% sodium hydroxide solution. The white masses so obtained were again recrystallized from chloroform, m. p. 157–158.°

Anal.—Calcd. for $C_{14}H_{16}N_2O$: C, 73.65; H, 7.07; N, 12.27. Found: C, 73.41; H, 7.22; N, 12.12.

The hydrochloride salt of α-aminomethyl-β-(1-naphthyl)propionamide was prepared by dissolving the compound in a small volume of absolute ethanol and adding an excess of anhydrous ether saturated with hydrogen chloride gas. The hydrochloride was recrystallized by dissolving it in the smallest possible volume of absolute ethanol, chilling the solution, and adding an excess of anhydrous ether. White crystals which melted at 243–244° were obtained.

Anal.—Calcd. for $C_{14}H_{17}ClN_2O$: N, 10.58; Cl, 13.39. Found: N, 10.78; Cl, 13.29.

SUMMARY

1. The ethyl ester and the amide of α-aminomethyl-β-(1-naphthyl)propionic acid have been prepared as potential oxytocic agents.

2. The intermediates ethyl α-cyano-β-(1-naphthyl)acrylate and α-cyano-β-(1-naphthyl)acrylamide have been prepared the former by a new procedure.

3. When subjected to pharmacological screening, the compounds failed to exhibit significant activity.

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The Influence of Gibberellic Acid on the Growth and Alkaloid Biogenesis in *Datura stramonium* Linné and in *Atropa belladonna* Linné*

By GORDON M. SMITH and LEO A. SCIUCHETTI

Gibberellic acid in concentrations of 100 and 1,000 p. p. m. was administered as an aqueous spray to the leaves and tops of *Datura stramonium* and *Atropa belladonna*. Characteristic effects on the growth of the treated plants are described. Significant increases in plant height were demonstrated. The fifty-four-day-old stramonium plants were harvested four weeks after the first treatment. Fresh and dry weight data indicated increases in total, leaves and tops, and stem weights, but decreased root weight. The twelve-week-old belladonna plants were harvested seven weeks following treatment. Appreciable decreases in leaves and tops and root dry weights were noted. However, this was accompanied by significant increases in stem weight. Stramonium plants receiving both a single and double spray treatment with each concentration of gibberellic acid showed decreases in the concentration of total alkaloids in the leaves and tops and in the stems. Increased alkaloid production was observed in the roots. The favorable effects on growth from the two-spray treatment of 100 p. p. m. of gibberellic acid resulted in significant increases in total alkaloid production. Significant decreases in the concentration of alkaloids in the plant organs of belladonna were noted. Further, the total alkaloid content of belladonna treated with gibberellic acid was about one-half that of the untreated plants.

NUMEROUS PAPERS have recently appeared reporting the various effects of gibberellic acid or the gibberellins on plant growth (1–7). Further, specific effects have been demonstrated in members of the *Solanaceae*. For example, Lang (8) has shown gibberellic acid effectively substitutes for the cold requirement of biennial *Hyoscyamus*. Also, evidence has been presented to indicate that an increase in cell division resulted from the application of gibberellins to biennial *Hyoscyamus niger* (9). However, the available literature has not indicated that any studies have been performed on the effect of this growth-inducing chemical on alkaloid biogenesis in the *Solanaceae*. The purpose of this study was to investigate, under controlled greenhouse conditions, the influence of gibberellic acid on the growth and alkaloid biogenesis in *Datura stramonium*. It was further decided to extend this line of investigation to a closely related plant, *Atropa belladonna*. With the latter it was decided

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to change the frequency of application of gibberellic acid and the environmental conditions of growth, *viz.*, growing the plants under field conditions.

EXPERIMENTAL

Procedure.—*Datura stramonium* plants used in this study were grown under carefully controlled greenhouse conditions. Seeds were germinated in culture flats containing soil composed of equal parts of sand, peat moss, and sterilized soil. Sixty-two sixteen-day-old seedlings were transplanted into 2 x 2-inch paper bands in flats containing soil composed of two parts sandy loam and one part peat moss. On July 15, 1957, the twenty-two-day-old seedlings which had attained a height of approximately 5.5 cm. were transplanted into individual clay pots (8 x 8 inches). On July 19, 1957, 60 of the potted plants were selected for this study and divided into three groups of 20 plants each. The first group constituted the control or untreated plants; the second were those treated with 100 p. p. m. of gibberellic acid (G. A.);[1] and the third were those treated with 1,000 p. p. m. of G. A. Freshly prepared aqueous solutions of G. A. were administered in the form of a spray to the leaves and tops of the treated plants. At the start of the third week, half of the plants in each of the treated groups received a second spray treatment with the aforementioned concentrations of G. A. The fifty-four-day-old plants were harvested four weeks after the first treatment.

The belladonna seeds were germinated in a manner similar to that for stramonium. On July 15, 1957, 100 two-week-old seedlings were transplanted into 2 x 2-inch paper bands in flats containing soil composed of two parts of sandy loam and one part of peat moss. On July 19, 1957, 84 of the banded plants were selected for this investigation and divided into three groups of 28 plants each. The plan for treating each group was similar to that for stramonium. The first treatment was administered to groups 2 and 3 at this time.

Ten days later the treated plants were given a second application of G. A. At this time the plants were transplanted into a prepared plot of ground in the drug garden north of the pharmacy building. The area chosen for this study was at the perimeter of a plot of ground in which digitalis plants had been planted. The light intensity in this area varied considerably due to the shade from the building and surrounding trees. The belladonna plants were spaced 2 feet apart in double rows. Each hill into which the belladonna was transplanted was fertilized by thoroughly working into the soil 15 Gm. of lime (calcium carbonate) and 20 Gm. of complete organic fertilizer.[2] Previous analysis of soil samples from this plot indicated that fertilization was desirable.

A third treatment with G. A. was given ten days later to the plants in groups 2 and 3. Ten plants were picked at random from each group and were harvested. This was seven weeks after the final treatment. The remaining plants were allowed to remain for further study.

During the harvest period each species was divided into three portions; leaves (with flowering and fruiting tops), stems, and roots. Fresh weights were promptly taken of each individual portion and the fresh material was immediately transferred to a hot-air circulating drier. The leaves and root portions were dried at a temperature of 48.5° for thirty hours while the stems were dried forty hours. Upon removal from the drier the plant parts were placed in a desiccator, allowed to attain room temperature, and then weighed. The portions were placed in airtight metal containers until subsequent pulverization into a No. 40 powder in a Wiley mill. The powdered material was then stored in airtight colored-glass containers until subsequent analyses for alkaloids were performed.

Growth Effects in Stramonium.—The plants were carefully observed daily for specific growth effects during the four-week treatment period. The height of each plant was taken at approximately the same time each day and was determined by measuring the distance in cm. from the base of the stem to the active growing tip of the plant. In general, after two weeks, the plants treated with G. A. demonstrated the following differences compared with the controls: increased height due primarily to increased stem elongation; leaves which were longer, thinner and slightly chlorotic; less succulent growth; increased rate of flowering and plant maturity. Similar effects have previously been reported in other plants (1–6, 11). At the end of the fourth week the plants receiving a two-spray treatment demonstrated the "gibberellin" effects previously indicated, but to a greater extent (Fig. 1). However, at this time, the plants receiving only one treatment with G. A. appeared to resemble more closely controls in growth characteristics with the exception that the leaves remained slightly chlorotic. It was further observed that plants treated with G. A. were more prone to insect attacks. This occurred in spite of the fact that all plants were

Fig. 1.—Effect in *Datura stramonium* after four weeks from a two-spray treatment with gibberellic acid. *A* = 1,000 p. p. m., *B* = 100 p. p. m., *C* = control.

[1] The gibberellic acid used in this study was supplied through the courtesy of Dr. Curt Leben, Eli Lilly & Co., Indianapolis, Ind.

[2] Organic Morcrop, Chas. Lilly Co., Seattle, Wash. (Analysis—5% of total nitrogen, 3% available phosphate, 2% available potash.)

dusted twice weekly with Maladusto 4D[3] (4% malathion dust).

The most characteristic effect induced in stramonium by the treatment with G. A. was increased stem elongation due mainly to an increase in length between the internodes of the stem (Fig. 1). This resulted in considerable increases in the height of the treated plants (Fig. 2). Compared with the controls, the plants treated with both concentrations of G. A. displayed increases in height of about 45% at the first week and approximately 36% at the second week. The group treated with a single spray of 100 p. p. m. of G. A. indicated increases in height of approximately 25% and 12% at the third and fourth weeks, respectively. Correspondingly, the plants treated with the higher concentration of G. A. (1,000 p. p. m.) showed increases of 26% and 13%, respectively. Significant increases in height were induced in those plants receiving a second spray of G. A. For example, at the lower concentration the increases were 56% and 35% at the third and fourth weeks, while at the higher concentration the corresponding increases were 51% and 49% (Fig. 2).

Growth Effects in Belladonna.—The belladonna plants treated with G. A. were somewhat taller than the controls at the time of transplantation (ten days after the first treatment). After the second treatment the treated plants grew more rapidly than the controls. Further, at this time it was observed that the plants treated with G. A. were more prone to insect attacks than the untreated, even though all plants in the drug garden were dusted twice weekly with 4% malathion. This characteristic was noted thereafter throughout the growth period. The growth effect from G. A. was much more pronounced after the third treatment. This was characterized by an immense elongation between the stem internodes and the formation of numerous lateral shoots at the internodes. Gray (6) has reported a similar effect in bean plants. The plants were much more spindly than the untreated and did not appear as healthy. From the fourth week following treatment until the plants were harvested the following growth characteristics were observed in the treated plants: they were spindly and vine-like; the leaves were slightly chlorotic, narrower, thinner, and less succulent; some of the younger leaves were crinkled and appeared to be blistered; the plants were taller; the onset of flowering was more rapid; and, in general, they did not appear as healthy as the controls (Fig. 3). In general, many of the gibberellin effects were similar to those displayed in *Datura stramonium*. Also Gray (6) has reported that the leaves of pepper plants became rough instead of smooth and tobacco leaf blades became more elongated, following a spray treatment with G. A.

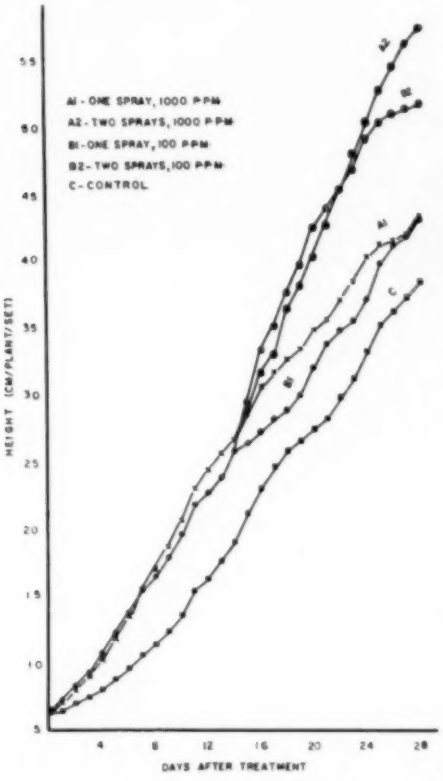

Fig. 2.—Increase in height of *Datura stramonium* by treatment with gibberellic acid.

[3] Manufactured by Miller Products Co., Portland, Ore.

Fig. 3.—Growth effects in *Atropa belladonna* seven weeks after treatment with gibberellic acid. *A* = 1,000 p. p. m., *B* = 100 p. p. m., *C* = control.

The most pronounced effect induced by the G. A. was increased stem elongation due primarily to an increase in the length between the internodes of the stems (Fig. 3). Significant increases in the height of the treated plants were observed from measurements taken at the fourth, sixth, and seventh week following the last treatment (Fig. 4). The plants treated with 100 p. p. m. of G. A. attained a height of approximately 312%, 224%, and 191% of the controls at the fourth, sixth, and seventh week, respectively. The corresponding figures for the plants treated with 1,000 p. p. m. of G. A. were 370%, 271%, and 241% (Fig. 4). The plants treated with the higher concentration of G. A. displayed the greatest increases. Phinney (2) has reported that the degree of response from gibberellin in corn seedlings was related to dosage, and Marth, *et al.* (4), have shown that many plants were 50–300% taller within three to four weeks following treatment with G. A.

Correlation with Fresh and Dry Weight Data.—In stramonium, significant increases in total weight were demonstrated from all the treatments, ranging

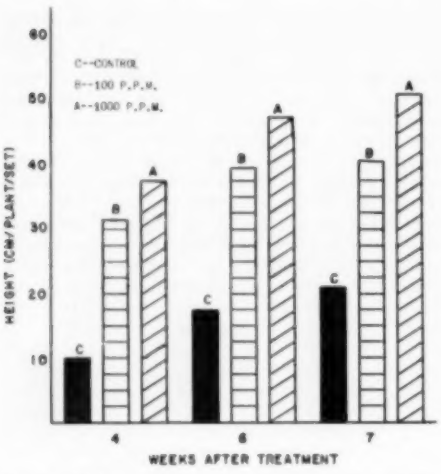

Fig. 4.—Height of *Atropa belladonna* after treatment with gibberellic acid.

from 120.5 to 159.7% of the controls (Table I). The plants receiving two treatments of 100 p. p. m. of G. A. displayed the largest increase (56.6%) in the leaf and top weights, while those receiving two sprays at the higher concentration displayed the smallest increase (14.1%). Significant increases in stem dry weights were shown from all the treatments. The stramonium plants treated with two sprays of G. A. demonstrated the largest increases. The largest increase (91.3%) was shown with the lower concentration of G. A. (Table I). On the other hand, the group receiving two treatments of the higher concentration indicated the greatest stem growth when leaf/stem ratios were employed. Decreases in root dry weights were demonstrated from all the treatments. The largest decrease (31%) was indicated by the plants receiving two treatments of the higher concentration. The inhibition of root growth by the G. A. was further demonstrated when comparing shoot/root ratios which were calculated from dry weight data.

With belladonna, significant decreases in leaves and tops and root dry weights were indicated from both treatments (Table II). The decreases were greater in the plants treated with the lower concentration of G. A. However, this was accompanied by significant increases in stem dry weights. The greatest increase was displayed by plants treated with the higher concentration of G. A. No significant changes in total dry weights were indicated. The leaf/stem ratio for the control group was 5.09 and that for the plants treated with the lower and higher concentrations of G. A. were 1.66 and 1.53. This further demonstrates the profound effect that G. A. had on stem growth. Inhibition of root growth was further indicated from the G. A. since the shoot/root ratios for both treatments were greater than the controls.

In both plants, when the dry weights of the shoot organs were divided by their fresh weights, the controls indicated the greatest loss of moisture. This confirms the observations made during the growth period that control shoots appeared more

TABLE I.—WEIGHTS OF STRAMONIUM PLANT PARTS (AV./PLANT/GROUP)

Treatment	No. of Plants	Total Weight				Leaves and Tops				Stems				Roots			
		Fresh, Gm.	Dry, Gm.	Dry/Fresh, %	Control Dry Wt., %	Fresh, Gm.	Dry, Gm.	Dry/Fresh, %	Control Dry Wt., %	Fresh, Gm.	Dry, Gm.	Dry/Fresh, %	Control Dry Wt., %	Fresh Gm.	Dry, Gm.	Dry/Fresh, %	Control Dry Wt., %
Control	10	29.8 ± 1.8	3.95 ± 0.2	13.3	...	14.7	1.98	13.4	...	9.9	1.38	13.9	...	5.2	0.58	11.2	...
100 p. p. m. G. A., One spray	8	31.0 ± 1.7	4.76 ± 0.3	15.4	120.5	15.9	2.52	15.8	127.3	10.5	1.77	16.9	128.3	4.7	0.47	10.0	81.0
100 p. p. m. G. A., Two sprays	8	41.6 ± 0.9	6.31 ± 0.2	15.2	159.7	20.8	3.10	14.9	156.6	15.8	2.64	16.7	191.3	5.0	0.56	11.2	96.6
1,000 p. p. m. G. A., One spray	8	34.5 ± 0.9	4.91 ± 0.4	14.2	124.3	17.6	2.55	14.5	128.8	12.7	1.90	15.0	137.7	4.3	0.46	10.7	79.3
1,000 p. p. m. G. A., Two sprays	8	35.0 ± 2.6	4.99 ± 0.4	14.3	126.3	16.2	2.26	14.0	114.1	16.1	2.33	14.5	164.5	2.7	0.40	14.8	69.0

TABLE II.—WEIGHTS OF BELLADONNA PLANT PARTS (AV./PLANT/GROUP)

Treatment	Total Weight			Leaves and Tops			Stems			Roots		
	Fresh, Gm.	Dry, Gm.	Control Dry Wt., %	Fresh, Gm.	Dry, Gm.	Control Dry Wt., %	Fresh, Gm.	Dry, Gm.	Control Dry Wt., %	Fresh, Gm.	Dry, Gm.	Control Dry Wt., %
Control	52.86	4.90	...	38.77	3.31	...	7.49	0.65	...	6.60	0.94	...
100 p. p. m. G. A.	40.81	4.45	90.8	23.84	2.39	72.2	12.22	1.44	221.5	4.75	0.62	66.0
1,000 p. p. m. G. A.	45.23	4.96	101.2	25.36	2.59	78.3	14.98	1.69	260.0	4.89	0.68	72.3

succulent. This suggests that more carbohydrates and/or proteins may have accumulated in the shoot organs of the treated plants.

Analysis for Alkaloids.—The dried plant parts, using pooled samples, were assayed for total alkaloids according to the Witt-Youngken method (10), substituting chloroform for benzene as the immiscible solvent. In stramonium, considerable decreases in the percentage of total alkaloids (expressed as scopolamine) were generally indicated in the leaves and tops and in the stems, while increases were shown in the root organs. The concentration of alkaloids in the leaves and tops decreased 30.3 and 27.4%, respectively, for plants receiving the single spray treatment of the higher and lower concentrations of G. A. These decreases were considered significant. Significant decreases, ranging from 21.7 to 48.3%, were found in the accumulation of alkaloids in the stems. The greatest decrease was exhibited by the group treated with a single spray of 100 p. p. m. of G. A. Considerable increases in total alkaloid production in the roots were shown from the treatments. The increases due to the two-spray treatments ranged from 41.7 to 50% and were considered significant. The group receiving a two-spray treatment of 100 p. p. m. of G. A. displayed the highest concentration of alkaloids in the roots, *viz.* 150% of the controls.

In belladonna, decreases in the concentration of alkaloids of all the organs were demonstrated from both treatments. For example, belladonna treated with 100 p. p. m. of G. A. contained approximately 57, 53, and 84% of the total alkaloids of the controls in the leaves and tops, stems, and roots, respectively. The corresponding figures for the plants treated with 1,000 p. p. m. of G. A. were about 55, 43, and 77%.

Total Plant Alkaloids.—It was further desired to make comparisons based on the total alkaloids per plant and per plant organ. The total alkaloids (calculated as scopolamine) were obtained by multiplying the dry weight of the plant organ by the per cent of alkaloids obtained from the alkaloid analyses and expressing the results in milligrams (Table III). The most favorable response on total alkaloid production in stramonium was induced by the two-spray treatment with 100 p. p. m. of G. A. This treatment caused a decrease in the concentration of alkaloids in the shoot organs. However, the favorable response on growth resulted in a 46% increase in total plant alkaloids.

Significant decreases in total alkaloids (calculated as hyoscyamine) were found in the belladonna plant, leaves and tops, and roots, whereas increases were observed in the stems (Table IV). In general, the total alkaloid content of the treated plants was about one-half that of the controls.

TABLE III.—TOTAL ALKALOID CONTENT OF *Datura stramonium*[a]

Treatment	Per Plant		Leaves and Tops		Stems		Roots	
	Alkaloids, mg.	Control, %	Alkaloids, mg.	Control, %	Alkaloids, mg.	Control, %	Alkaloids, mg.	Control, %
Control	9.91	...	6.83	...	2.80	...	0.28	...
100 p. p. m. G. A., One spray	8.52	86.0	6.40	93.7	1.86	66.4	0.26	92.9
100 p. p. m. G. A., Two sprays	14.46	146.0	9.86	144.5	4.20	150.0	0.40	142.9
1,000 p. p. m. G. A., One spray	8.70	87.8	6.22	91.1	2.22	79.3	0.26	92.9
1,000 p. p. m. G. A., Two sprays	10.75	108.6	6.78	99.3	3.70	132.1	0.27	96.4

[a] Alkaloid content for plant parts calculated from dry wt. and alkaloid analysis data; total plant alkaloid = leaves and tops + stems + roots. All data were based on av./plant/group.

TABLE IV.—TOTAL ALKALOID CONTENT OF BELLADONNA[a]

Treatment	Per Plant		Leaves and Tops		Stems		Roots	
	Alkaloids, mg.	Control, %	Alkaloids, mg.	Control, %	Alkaloids, mg.	Control, %	Alkaloids, mg.	Control, %
Control	19.05	...	12.25	...	2.76	...	4.04	...
100 p. p. m. G. A.	10.29	54.0	4.59	37.5	5.25	117.8	2.45	60.6
1,000 p. p. m. G. A.	10.05	52.8	4.88	39.8	3.11	112.7	2.06	51.0

[a] Alkaloid content for plant parts calculated from dry wt. and alkaloid analysis data; total plant alkaloid = leaves and tops + stems + roots. Based on av./plant/group.

DISCUSSION

Characteristic responses were induced in *Datura stramonium* and *Atropa belladonna* by the spray treatments with G. A. Some of these responses have been reported in *Hyoscyamus niger* (8), pepper, tomato, and tobacco (6), and in various other plants (11). The most profound effect was the increased height of the treated plants due to increased stem elongation. Belladonna responded to this gibberellin effect to a greater extent than did stramonium. Further, it was observed that plants treated with G. A. were more prone to insect attack and did not appear as healthy and sturdy as the untreated plants. With belladonna, the treated plants required more labor in tying the plants to stakes in order to keep them upright. This was necessary because of the greatly increased number of axillary shoots and the vine-like and spreading-type of plant growth. These factors would present serious obstacles to the commercial cultivation of belladonna.

Different results between the two species regarding dry weights were induced by the treatments. In stramonium, significant increases in shoot dry weights were generally observed, accompanied by decreases in root dry weights. In belladonna, significant reductions in the dry weights of the leaves and tops and the roots were offset by significant increases in the dry weights of the stems. This resulted in slight but insignificant changes in the total dry weight of the treated plants. Inhibition of root growth was demonstrated for both species as indicated by reduced weights and increased shoot/root ratios. In the case of belladonna, this factor accompanied by no significant increase in total dry weight poses a serious limitation for the practical use of G. A. under conditions similar to this experiment.

Of more importance from a commercial aspect was the profound effect that G. A. had on alkaloid formation and accumulation in the plants. It was found in stramonium that a two-spray treatment with 100 p. p. m. of G. A. resulted in a 46% increase in total plant alkaloids. Increasing the concentration of G. A. was less beneficial, while the one-spray treatment of both concentrations proved to be unfavorable. On the other hand, treated belladonna plants contained about one-half the total amount of alkaloids as did the controls. These inconsistencies could be attributed to generic differences. However, it would appear that an optimum condition involving concentration and frequency of application is necessary for maximum beneficial effects. This optimum condition is now being investigated. Further, observations made during the growth of both species and from a study of the data suggest that changes in the carbohydrate and/or protein metabolism were induced by G. A. Research is now being directed toward a study of this possibility.

It was noted generally that in both species the concentration of G. A. was not critical to certain effects. There were some differences due to the strength of G. A. employed on increased height, fresh and dry weights, alkaloid concentration, and total alkaloid content. However, these differences were not considered significant. The unfavorable effect of G. A. on the alkaloid production in belladonna indicates that the concentrations of G. A. employed in this study were toxic. It is altogether possible that favorable responses in both growth and alkaloid production may have been induced with lower concentrations of G. A.

From this study the following similarities were observed in both species: the treated plants indicated significant increases in stem growth and inhibition of root growth; the treated plants appeared to lack natural resistance to insect attack; and, the shoots of the treated plants were shown (by observation and other data) to be less succulent than untreated plants. On the other hand, some differences were noted between the two species. The increases in height and stem growth were much greater in belladonna and the decreases in the alkaloid content of this species were greater. It should be pointed out that both plants were treated with similar concentrations of G. A. by spraying onto the leaves and tops. On the other hand, the following factors were different; the environmental conditions, the frequency of treatments, and time interval between the following treatments, the age of the plants. Of these factors, it would appear that the number of treatments and the time interval between and following treatments would be important considerations. The observations that the treated plants had decreased resistance to insects, were less succulent, and had less root growth suggest the possibility that essential nutrients required for normal plant metabolism and growth were being used for the increased stem growth. The decreased alkaloid content of *Atropa belladonna* and, in some cases, of *Datura stramonium*, further suggests that some of the nitrogenous material normally used for the biosynthesis of alkaloids was diverted toward increased stem growth and other metabolic pathways.

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Antibacterial Activity of the Heartwood of *Haematoxylon braziletto**

By ROBERTSON PRATT and YOKO YUZURIHA

Aqueous extracts of the heartwood of the Mexican plant, *Haematoxylon braziletto*, contain a substance which, in appropriate concentrations, is bactericidal for *Salmonella typhosa* and *Micrococcus pyogenes* var. *aureus* and is bacteriostatic for *Escherichia coli*. The active principle has not been unequivocally identified but it is suggested that the antibacterial action is due to the pigment, brazilein, or to its precursor, brazilin, or to some derivative(s) of these. Neither the unpigmented sapwood nor the bark of the plant contains the active principle. Hematoxylin, which is derived from the heartwood of the related plant, *H. campechianum*, and which contains pigments that are closely related chemically to those of *H. braziletto*, has similar antibacterial activity in similar concentrations.

THE HISTORY of pharmacy and medicine is replete with the wisdom as well as the folly of folklore, and a number of our most respectable drugs can be traced to very early beginnings in the empirical treatment of infectious, functional, or mental illnesses. Antibiotics, hormones, and tranquilizers are but three examples of classes of recently rediscovered drugs which, in the form of crude preparations, have been used therapeutically for centuries.

Therefore, our curiosity was aroused by receipt of a letter referring to *Haematoxylon braziletto* as follows:[1]

"... While doing field work in the Gulf of California and later on the mainland of Sonora, I found the people there using this plant for medicinal purposes. A sliver of wood dropped into a glass of water very quickly produces a pink solution strongly resembling permanganate of potash. The potion is then drunk as a remedy for "whatever ails you." I also found them placing this wood in drinking troughs of their chickens and, in some districts, in the water of the rural schools.

"... My question is, then, have the natives employed a drug for medicinal purposes where we have remained unaware of such value and used it merely in microtechnics?"

H. braziletto is limited in distribution to Lower California and adjacent states on the mainland of Mexico where, according to Gentry (1), it is common on mesas and arid hilltop slopes at elevations from 800 to 2,500 feet in Sonora and Chihuahua. At lower elevations it attains the size of a small tree but at higher levels (about 1,800 feet) it forms "low dense, hemispherical shrubs" occasionally so dense as to form "impenetrable thickets." The local names, brazilwood tree and brazil bloodwood tree, refer to the deep-red heartwood which Gentry notes is used to prepare a "light-red dye or ink" and also in fashioning rosary crosses. Martinez (2) adds the information that a decoction of the wood is used for "washing teeth." The native Indians call the plant "huchachago."

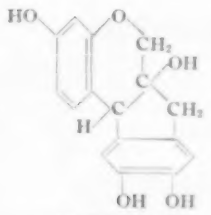
The local name, "palo de brazil," apparently has no geographic connotation concerning source, but rather is said to be derived from the word "braza," signifying "glowing like fire." Thus, the same common name is applied to a number of soluble-pigment-bearing red woods produced by different species of leguminous trees that grow in the East Indies, South and Central America, and Africa (3). Therefore, the plant is not to be confused with its relatives, *Caesalpinia echinata*, the brazilwood commonly found in the literature, or *C. brasilensis* and *C. crista*, the sources of brasiletto (4). However, all four plants are closely related botanically and chemically to each other and to *Haematoxylon campechianum*, the source of logwood and of the stain hematoxylin.

The red heartwood of *H. braziletto*, *C. echinata*, and *C. brasilensis* contains the water-soluble substance, brazilin (I) which upon mild alkaline oxidation or exposure to air yields the pigment red brazilein (II).

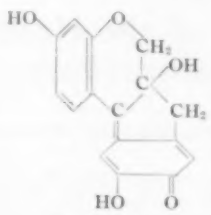
Search of the literature failed to reveal any reference to antibacterial action of constituents of *H. braziletto* and only inferential evidence concerning its relatives in the genera *Haematoxylon* and *Caesalpinia*. The 1899 edition of the U. S. D. (4) stated that logwood is "well adapted to the treatment of that relaxed condition of bowels which is apt to succeed *Cholera infantum*" and ascribed the effect to astringent action. The extract, which Hobbs' Botanical Handbook of 1876 (5) listed as an astringent tonic, was official as *Extractum Haematoxyli* in the National Formulary (6) until 1936. The isolated compound, hematoxylin has been reported to be "very feebly antiseptic" (7). The true brazilwood (*C. echinata*) and other similar woods, e. g., brasil-

* Received June 2, 1958, from the University of California, School of Pharmacy, San Francisco 22.

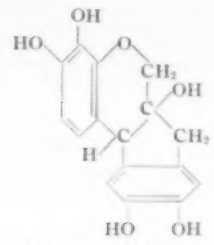
[1] From Loye H. Miller, Professor of Biology, Emeritus, University of California at Los Angeles.



Brazilin (I)[2]



Brazilein (II)[2]



Hematoxylin (III)[2]

etto, sappanwood, and Nicaragua wood produced by other species of *Caesalpinia*, were once used medicinally but by the latter part of the nineteenth century were considered inert, and their medico-pharmaceutical use was limited to coloring tinctures, etc. (4), and to preparation of mouthwashes and gargles (8).

EXPERIMENTS AND RESULTS

The first experiments were designed to simulate the native use of the red heartwood of *H. braziletto*. Small chips (total weight 1.0, 0.6, 0.4, and 0.1 Gm., respectively) were placed in separate test tubes, each containing 10 ml. of tap water. The pH of the water was 4.0, and addition of the wood caused no change in pH, even after several days. The tubes were inoculated with a standard loopful of an active culture of *Salmonella typhosa* ATCC 6539. Duplicate tubes were similarly inoculated with *Escherichia coli*, ATCC 6522, and all tubes were incubated for eighteen hours at 37°. Each series was set up in duplicate. Parallel tubes were treated with chips of the sapwood. Results are shown in Table I.

TABLE I.—TURBIDITY OF TAP WATER CONTAINING WOOD OF *Haematoxylon braziletto* AND INOCULATED WITH *S. typhosa* AND *E. coli* (INCUBATION: 18 HOURS AT 37°)

Amt. of Wood, Gm	*S. typhosa*		*E. coli*	
	Heartwood	Sapwood	Heartwood	Sapwood
None (Control)	+++	+++	+++	+++
0.1	+++	+++	+++	+++
0.4	Clear	+++	+++	+++
0.6	Clear	+++	+++	+++
1.0	Clear	++	+	+++

[2] The structure of brazilin (I) has been established with certainty; structures shown for brazilein (II) and hydroxybrazilin hematoxylin (III) are considered the most probable (3).

No organisms developed when transfers were made from the clear tubes into nutrient broth and incubated at 37°, and it was concluded that the heartwood contains a water-soluble constituent which, in appropriate concentration, is bactericidal—not merely bacteriostatic—for *S. typhosa*. The results suggested that there might be a sound basis for the empirical native use of the heartwood and prompted more rigorous tests.

Ten grams of the granulated heartwood was packed carefully into a small percolator and 50 ml. distilled water was added. After twelve hours of maceration, the percolate was removed slowly, shell frozen in a suitable vessel, immersed in a mixture of dry ice and acetone, and then lyophilized. Complete drying usually required twelve to fifteen hours of lyophilization. The resultant dry residue consisted of orange-salmon-pink, fluffy flakes that were soluble in distilled water to the extent of 33 mg./ml. Ten grams of heartwood yielded 120 to 130 mg. of dry residue from the first percolate.

Difco beef extract broth (14.9 ml.) containing different amounts of the dissolved extract was inoculated with 0.1 ml. of a culture of *S. typhosa*[3] and the cultures were incubated at 37° in inclined roller tubes rotating at a rate of 5 r. p. m. Periodically, the transmittance of the tubes (run in duplicate in each experiment) was determined by means of a Lumetron. In some experiments, hematoxylin[4] and brazilin[5] were studied comparatively with the wood percolate. Because of differences in color of broth containing different concentrations of extract or of the stains, it was necessary to run a separate uninoculated standard for each concentration of each product. The culture medium (pH 6.8) was well buffered, and even the highest concentration of extract or of the stains did not change the pH. Duplicate experiments were performed with *E. coli* and with *Micrococcus pyogenes* var. *aureus*.

Averaged results of several experiments are presented in Table II. Tubes with an optical density of 0.0 to 0.02 are visually clear. Thus, using visual criteria, one would consider the end point of both the crude extract and of hematoxylin to be between 0.25 and 0.5 mg./ml. for *S. typhosa* and between 0.125 and 0.25 mg./ml. for *M. pyogenes* var. *aureus*. Brazilin was slightly more antagonistic to the bacteria, the end point against *S. typhosa* and *M. aureus* being one tube higher in the dilution series. Failure of organisms to develop when transfers into drug-free broth were made from tubes with a reading of 0.01 or less suggests that the compounds were cidal, not merely static, in the stated concentrations.

E. coli was more sturdy than either of the other organisms and could neither be killed nor permanently inhibited even at the highest concentration tested (0.5 mg./ml.), although growth was suppressed for several hours. Cultures of this organism remained visually clear (o. d. = 0.02 or less) for three hours after inoculation into broth containing 0.0625 mg. of *H. braziletto* extract or of brazilin per ml., and for six hours when inoculated into

[3] Eighteen-hour culture adjusted to a transmission of 47% as determined in Pyrex tubes (18 mm. i. d.) in a Lumetron Model 402 EF equipped with a neutral filter.

[4] National Aniline Division of Allied Chemical and Dye Corporation, certification No. NH3.

[5] Histological stain from Hartman-Leddon Company, C. I. No. 1243.

TABLE II.—PER CENT TRANSMISSION AND OPTICAL DENSITY OF BROTH CULTURES OF THREE SPECIES OF BACTERIA INCUBATED FOR TWENTY-FOUR HOURS AT 37° IN THE PRESENCE OF *H. braziletto* HEARTWOOD, EXTRACT OF HEMATOXYLIN, AND OF BRAZILIN

Treatment	*S. typhosa*		*E. coli*		*M. pyogenes* var. *aureus*	
	T, %	O. D.	T, %	O. D.	T, %	O. D.
Control	5.0	1.30	4.6	1.34	2.0	1.70
Extract, mg./ml.						
0.5	98.1	0.01	Not tested		100.0	0.00
0.25	17.0	0.77	2.3	1.64	100.0	0.00
0.125	8.9	1.05	2.9	1.54	71.1	0.15
0.0625	2.5	1.60	2.9	1.54	31.9	0.50
Hematoxylin, mg./ml.						
0.5	96.7	0.01	2.6	1.59	98.6	0.01
0.25	19.5	0.71	2.5	1.60	99.0	0.00
0.125	14.7	0.83	5.5	1.26	17.2	0.76
0.0625	16.3	0.79	5.5	1.26	11.3	0.95
Brazilin, mg./ml.						
0.5	100	0.00	5.0	1.30	100	0.00
0.25	100	0.00	3.9	1.41	100	0.00
0.125	33.3	0.48	3.9	1.41	100	0.00
0.0625	17.8	0.75	4.2	1.38	97.4	0.01
0.03125	Not tested		Not tested		91.2	0.04
0.015625	Not tested		Not tested		10.6	0.97

broth containing the same concentration of hematoxylin. Control cultures usually showed some visible turbidity at the end of the first or second hour.

M. pyogenes var. *aureus* was the most easily affected of the organisms that were studied. Even after twenty-four hours of exposure to the lowest concentration of extract or of hematoxylin tested (0.0625 mg./ml.) growth in the experimental cultures was visibly considerably less than in the controls. The effect was particularly pronounced in cultures exposed to brazilin which, at that concentration, completely suppressed growth and almost completely suppressed it at 0.03125 mg./ml.

In agar-incorporation tests neither the extract (in concentrations from 0.25 to 4.0 mg./ml.) nor hematoxylin (in concentrations from 0.25 to 1.0 mg./ml.) evidenced antimycotic activity against *Penicillium notatum* or *Monilia fructigena*. However, the extract, at 2 mg./ml., prevented growth of *Trichophyton mentagrophytes* for four days and partially inhibited growth for ten days. At a concentration of 4 mg./ml., the extract completely suppressed growth of the organism for ten days, at which time the tests were terminated. Hematoxylin at levels from 0.25 to 1.0 mg./ml. partially inhibited growth of *T. mentagrophytes* for four days but not for longer test periods.

Successive extracts were made from each portion of granulated heartwood by adding 50 ml. distilled water to the percolator immediately after the previous percolate had been removed. Usually the yield from each of the first two percolates from a given aliquot of wood was between 120 and 130 mg. of dry, fluffy lyophilate. With succeeding percolations, the yield dropped until the 14th to 15th percolates yielded only 8 to 10 mg. of lyophilate. The total recovery in 15 extractions of 10 Gm. of heartwood was 600 to 650 mg. of lyophilate for a yield of 6 to 6.5%. There were no obvious physical differences in the appearance, either gross or microscopic, of the products obtained from the first and last extractions. But with succeeding extracts, beyond the second or third, there was a decrease in water solubility and in antibacterial activity. Lyophilates from the first two extractions of a given sample were active; those from fourth or later extractions were inactive.

Spectrophotometry failed to reveal significant differences between solutions of the active and inactive extracts, whether they were examined in acid, neutral, or alkaline media. Typical curves for solutions at pH 6.0 showed a primary absorption peak in the ultraviolet at 252 mμ and a secondary one at 285 mμ. A broad primary peak occurred in the visible range at 440 to 450 mμ and a secondary peak occurred at 520 mμ. There was no correlation between the order of the curves and the activity of the extracts. For example, in a typical experiment with a strongly bactericidal solution, a completely inactive solution, and one with intermediate activity, the absorbance in the ultraviolet range, in decreasing order, was active solution, inactive solution, and intermediately active solution. But in the visible wavelengths the decreasing order of absorbance was inactive solution, most active, and intermediately active. And in the infrared region, the curves of all three solutions were virtually identical, both qualitatively and quantitatively. Although the extinction coefficients at a given wavelength were different for the different solutions, the ratios of primary and secondary peaks in the ultraviolet and again in the visible were the same for the three solutions.

Solutions of brazilin exhibited only one peak in the ultraviolet, namely, at 285 mμ, and there was no absorbance in the visible. The absorbance of a 1.0 mg. % aqueous solution (pH 6.0) of brazilin at that wavelength was 0.223 with 1 cm. path length. The corresponding value for the active extracts was 0.220. There was little change upon acidification to pH 1.5 with HCl, the corresponding values at 285 mμ being 0.214 for brazilin and 0.216 for the extracts. In alkaline media (pH 10.0) a broad band with a distinct peak at 520 mμ was prominent. The absorbance at that wavelength was 0.948 for brazilin (1 mg. % at 1 cm. path length) and 0.191 for the active extracts. However, the compounds

were unstable in alkaline solution. Upon restoration of the original pH, the pattern of absorption was quite different from that obtained originally and the curves produced upon subsequent realkalinization were entirely different from those just described.

Paper chromatography was disappointing. All of the extracts yielded two spots; one which was easily visible and one which was only faintly visible in white light but both of which fluoresced brilliantly under ultraviolet irradiation. These pairs of spots had identical R_f values in the bacteriologically active, moderately active, and inactive extracts. Ultraviolet scanning failed to reveal any additional spots not detectable in white light. Similar spots, of different intensity and fluorescence but with identical R_f values, were produced by solutions of hematoxylin. The pairs of spots in all the preparations increased in intensity upon standing in air, and they were attributed to the pigments. Brazilin produced a single spot which appeared to be qualitatively identical with one of the spots obtained with the extracts, but much more intense. Tests with mixtures of brazilin and the extracts in varying proportions failed to separate the spots, suggesting that the extracts contained small amounts of brazilin.

DISCUSSION

The failure of spectrophotometry to reveal qualitative or systematically graded quantitative differences among bacteriologically active, moderately active, and inactive solutions of the extracts suggests that the action of the effective solutions was not due directly to the pigments they contained and raises the possibility that the activity was due to some other compound or compounds. However, although extensive chromatographic studies were not made, the trials which were performed failed to provide evidence of such a compound(s) and seemed to indicate qualitative chromatographic equivalence of the active, moderately active, and inactive extracts. The similarity of chromatographic patterns developed by solutions of bacteriologically active and inactive extracts and of hematoxylin suggests again that the activity was not associated with the pigments.

On the other hand, despite the above evidence, it is difficult to suppress the idea of a relationship between the pigment and the observed antibacterial action. Tending to point to such a connection are the facts that (*a*) extracts prepared from the unpigmented sapwood and bark were completely inactive against the test organisms; (*b*) the aqueous extracts prepared from the heartwood would be expected to contain brazilin; (*c*) despite some differences, aqueous solutions of brazilin and of the extracts had spectrophotometric similarities; (*d*) brazilin and hematoxylin have closely related phenolic structures (the latter being simply hydroxybrazilin); (*e*) many phenols are known to be antibacterial; and (*f*) there was approximate concordance between the end point concentrations of the most active solutions of the *H. braziletto* extracts and of hematoxylin and of brazilin for inhibition of the test organisms. The latter point could, of course, be simply a matter of coincidence. But, in view of the close structural similarity of brazilin and hematoxylin, and of their products, brazilein and hematein, this does not seem plausible.

Brazilin and hematoxylin and their oxidation products, brazilein and hematein, respectively, are extremely sensitive to exposure to light and air and to slight alterations in pH, particularly in alkaline or near-alkaline media, and to other environmental conditions. It seems possible, therefore, that they may exist in several isomeric forms which might not be distinguishable by spectrophotometric or chromatographic methods but which might, nevertheless, differ in their biologic properties. Therefore, it is suggested that the observed antibacterial activity was, in fact, due to specific isomeric form(s) of the pigment. The decrease in antibacterial activity of successive extracts prepared from a given sample of wood and the variation seen in a particular extract, e. g., first, second, etc., from different samples of wood would, according to this working hypothesis, be ascribed to the existence of different proportions of bacteriologically active and inactive isomers in the different preparations.

SUMMARY

1. In some areas of Mexico chips of the wood of the native plant, *Haematoxylon braziletto*, are added to the drinking water and the infusion is used empirically as a tonic (and anti-infective?) in some enteric disorders.

2. The heartwood of this plant contains a water-soluble compound which, in appropriate concentrations, is bactericidal for *Salmonella typhosa* and *Micrococcus pyogenes* var. *aureus* and temporarily inhibits the growth of *Escherichia coli*.

3. Solutions of the stains, brazilin and hematoxylin, derived from related plants, have a similar effect.

4. It is suggested that the activity of the extracts is due to one or more isomeric forms of brazilin and/or its oxidation product, brazilein.

5. Extracts prepared from the sapwood and from the bark of *H. braziletto* were inactive antibacterially.

6. None of the preparations exhibited antifungal activity against *Monilia fructigena* or *Penicillium notatum*, although both hematoxylin and *H. braziletto* temporarily retarded growth of *Trichophyton mentagrophytes*.

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A Note on Condensation Reactions of N-Guanidino-*p*-benzoquinone Imine Hydrazine Derivatives*

The *in Vivo* Antibacterial Activity of One Derivative

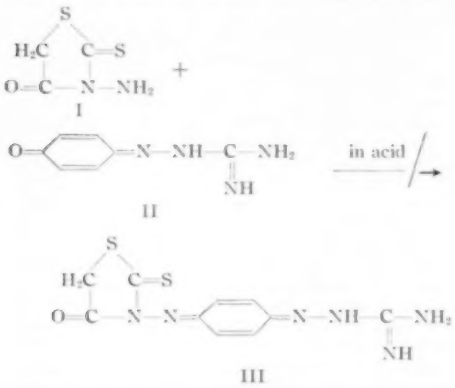
By RICHARD E. STRUBE and CHARLES LEWIS

The condensation product of 3-hydrazino-4-amino-5-mercapto-1,2,4-triazole with N-guanidino-*p*-benzoquinone imine was active in mice infected with *S. hemolyticus*. 3-Aminorhodanine, methyl dithiocarbazinate, 2-methylthiosemicarbazide and a number of 4-amino-1,2,4-triazole derivatives did not condense with N-guanidino-*p*-benzoquinone imine.

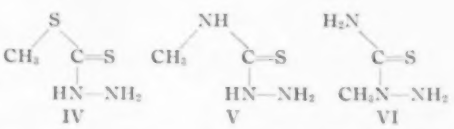
A PREVIOUS PUBLICATION from this laboratory described the condensation of 3-aminorhodanine (I) with aromatic and heterocyclic aldehydes (1). It was shown that in an acid medium reaction occurred with the amino group and not with methylene group of 3-aminorhodanine. Domagk's discovery of the activity of certain quinone derivatives in mice against Streptococci, in particular the compounds formulated below (2, 3), prompted the study of the reaction of 3-aminorhodanine with N-guanidino-*p*-benzoquinone imine (II) in the

X = S, O, or NH

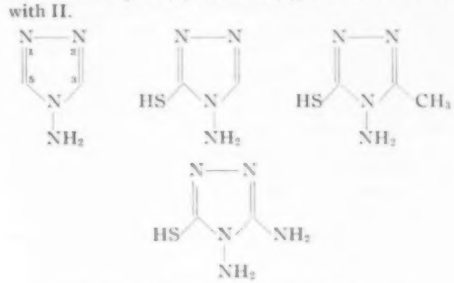
expectation of obtaining compound III. However, all attempts to make this compound were



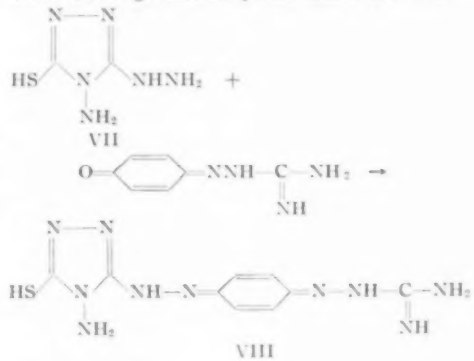
unsuccessful. The condensation of II with methyl dithiocarbazinate (IV), an open-chain analog of I, also failed. The desired condensation occurred readily with 4-methylthiosemicarbazide (V), a nitrogen analog of IV. These few experimental results suggested that failure of condensation of 3-aminorhodanine was due to the effect of the ring sulfur atom. However, additional experiments showed that this was not the only factor involved. In general, the disubstituted hydrazino group >N-NH$_2$, which is also present in 3-aminorhodanine, is unable to condense with II. This assumption was supported by the study of the condensation reaction of a few other disubstituted hydrazine derivatives. As already mentioned, condensation occurred with the monosubstituted hydrazine (V) but the disubstituted analog (VI) (4) did not condense. A number of 4-amino-1,2,4-triazoles, such as 4-amino-1,2,4-triazole (5), 4-amino-5-mercapto-1,2,4-triazole (6), 3-methyl-4-amino-5-mercapto-1,2,4-triazole (7), and 3,4-diamino-5-mercapto-1,2,4-triazole (8), did not condense with II.



IV V VI



A condensable triazole was obtained by introducing a monosubstituted hydrazine group. This compound, 3-hydrazino-4-amino-5-mercapto-1,2,4-triazole (VII), could be prepared in higher yields than reported in the literature (5, 9) by allowing diethylxanthate to react with an excess of hydrazine. Condensation of this thiazole with II gave compound VIII. Although this compound had a low order



VII VIII

* Received May 9, 1958, from the Research Division, The Upjohn Company, Kalamazoo, Mich.

The authors wish to thank Dr. J. B. Wright and Mr. D. A. Lyttle of the Department of Chemistry of The Upjohn Company for the preparation of a few thiazole derivatives.

TABLE I.—COMPARISON OF *in Vivo* ANTIBACTERIAL ACTIVITIES[a] OF COMPOUND VIII WITH N-GUANIDINO-N'-THIOUREIDO-*p*-BENZOQUINONE DIIMINE NITRATE IN INFECTED MICE

Organism	Compound VIII, Mononitrate			Domagk's Compound	
	Oral	i. p.	s. c.	Oral	i. p.
S. hemolyticus C203	87	*ca.* 80	*ca.* 320	<40	<25
D. pneumoniae I Felton	>800	...	>320	>80	...
K. pneumoniae A-D	>800	>80	...	>80	>25
M. aureus 284	>800	...	...	...	...
P. multocida 449	>800	...	>320	50–100	...
Tolerated dose	>800	80–100	>320	50–100	25

[a] Expressed as median protective doses in mg./Kg.

of activity against a series of bacteria in the agar-dilution plate test method, it was active in mice against *S. hemolyticus* when administered by the oral, subcutaneous, or intraperitoneal route.

EXPERIMENTAL

3 - Hydrazino - 4 - amino - 5 - mercapto - 1,2,4-triazole.—Thirty cubic centimeters of hydrazine (95%), 37.5 cc. of hydrazine hydrate, and 7.5 cc. of water were placed in a 250-cc., 3-neck, round-bottom flask provided with a stirrer, a reflux condenser, a dropping funnel, and a gas inlet tube for nitrogen. The system was swept out with a stream of nitrogen which was allowed to run until the product was isolated. The flask was placed in a water bath maintained at 60° and 75 Gm. (0.50 mole) of diethylxanthate was added to the well-stirred solution over a period of thirty minutes. Ethyl mercaptan, evolved rapidly, was absorbed by traps, one containing an excess of 30% sodium hydroxide, and the following one containing mercuric chloride and cadmium carbonate. The reaction mixture was stirred for an additional thirty minutes at 60°. During this period the condenser was maintained at 30° to allow ethyl mercaptan to escape. The flask was heated for one and one-half hours on a steam bath, with stirring. After cooling to room temperature, 250 cc. of water was added. The pH was adjusted to 6 with concentrated hydrochloric acid (35 cc. required) and the total volume was adjusted to 400 cc. with water. The mixture was heated to boiling and filtered while hot. The solid was washed with boiling water made slightly acid with hydrochloric acid. The solid was dried *in vacuo* at 60°. The yield was 25.1 Gm., m. p. *ca.* 221°, decompn. This material was purified by treating with 200 cc. of *N* NaOH plus 60 cc. of water. The insoluble material was removed by filtration and the clear filtrate was acidified by the addition of 18 cc. of glacial acetic acid. The solid formed was collected and washed with water and alcohol. After drying *in vacuo* at 60°, the white product weighed 24.3 Gm. (66.6% yield), m. p. 222–228°, decompn.

Condensation Product VIII.—N-Guanidino-*p*-benzoquinone imine (8.20 Gm., 0.05 mole) was dissolved in a solution of 65 cc. of water and 14.5 cc. of concentrated hydrochloric acid at room temperature. To this solution was added a solution of 3-hydrazino-4-amino-5-mercapto-1,2,4-triazole (7.26 Gm., 0.0497 mole) in 118 cc. of water and 25.4 cc. of concentrated hydrochloric acid at room temperature. The mixture was filtered after forty-eight hours and the dark red solid was washed, in two portions, with 100 cc. of water made acid with 2 cc. of concentrated hydrochloric acid. The product was purified by recrystallization from a minimum of boiling, slightly acid water (approx. 1,400 cc. required, prepared by adding 20 cc. of concentrated hydrochloric acid to 1,600 cc. of water). After drying *in vacuo* over sodium hydroxide at room temperature, 9.8 Gm. (60%) of compound VIII was obtained as the hydrochloride.

Anal. Calcd. for $C_9H_{14}ClN_{10}S$: C, 32.88; H, 3.98; Cl, 10.78; S, 9.75. Found: C, 32.99; H, 3.81; Cl, 10.67; S, 9.80.

The mononitrate of compound VIII was prepared in a somewhat similar way using nitric acid instead of hydrochloric acid.

Antibacterial Activity in Mice.—The procedure for testing compounds in infected mice has been described previously (10). Male CF-1 mice were infected with 100 LD_{50}'s of the organism to be studied and the infected animals were treated on three successive days with the compound. At the end of seven days, the efficacy of the compound was judged by the mortality ratio of the treated animals as compared to the mortality ratio of an untreated group.

Since compound VIII (nitrate) is rather insoluble in water, it was suspended in 0.25% Methocel.[1] The desired drug level was administered either subcutaneously or intraperitoneally in 0.2 cc. volume, or orally by intubation in 0.5 cc. volume.

Preliminary tests demonstrated that noninfected mice tolerated daily subcutaneous and oral doses of 320 mg./Kg. and 800 mg./Kg., respectively, without showing gross signs of toxicity. Pertinent results observed during the study of this compound are summarized in Table I. The original compound of Domagk (2) was also studied and the data are included for comparative purposes.

The data in Table I show that compound VIII was not toxic and that it protected *S. hemolyticus* infected mice when given by either the oral, subcutaneous, or intraperitoneal route. Its activity can best be demonstrated when given via the oral route. The activity of Domagk's compound as reported by him has been confirmed.

SUMMARY

1. 3 - Hydrazino - 4 - amino - 5 - mercapto - 1,-2,4-triazole condensed with N-guanidino-*p*-benzoquinone imine.

2. The condensation product is active in mice against *S. hemolyticus* when administered by the oral, subcutaneous, or intraperitoneal route.

[1] Dow Chemical Company, Midland, Mich.

3. 3-Aminorhodanine, methyl dithiocarbazinate, 2 methylthiosemicarbazide, 4-amino-1,2,4-triazole, 4 - amino - 5 - mercapto - 1,2,4 - triazole, 3 - methyl-4-amino-5-mercapto-1,2,4-triazole, and 3,4-diamino-5-mercapto-1,2,4-triazole did not condense with N-guanidino-*p*-benzoquinone imine.

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Book Notices

Analytical Abstracts. A monthly publication of The Society for Analytical Chemistry, 14 Belgrave Square, London, S. W. 1., England.

Analytical Abstracts, first published in January 1954 on the cessation of publication of *British Abstracts C, Analysis and Apparatus*, covers the analytical literature of the world.

It contains upwards of 4,000 abstracts per annum prepared by abstractors who are experts in particular fields of analytical chemistry. Many abstracts are prepared by chemists in the countries of publication of the original papers. Analysts will appreciate the advantage of having all abstracts dealing with their subject in a single publication.

Analytical Abstracts can be obtained from January 1, 1959 for $14 per annum postage-free including index. Some earlier volumes are still available. The subscription rate including *The Analyst* is $23.52 per annum postage-free, including both indexes. *Analytical Abstracts* is also available printed on oneside of the paper, so that abstracts can easily be incorporated into existing reference systems. Price per annum without index $15.05; with index $18.48.

How to Live With Diabetes. By HENRY DOLGER, M.D. and BERNARD SEEMAN. W.W. Norton & Co., Inc., New York, 1958. 192 pp. 14 x 21 cm. Price $3.50.

A book designed for diabetics and those concerned with the disease. The major emphasis is on the person with diabetes, and covers much information which he should have available. The book can conscientiously be recommended by pharmacists to diabetics.

Experimental Pharmacodynamics. 1958 rev. By T. KOPPANYI and A. G. KARCZMAR. Burgess Publishing Company, Minneapolis, 1958. xvi + 258 pp. 21 x 27.5 cm. Price $5.50 Paperbound. Printed by photo offset.

This brief outline attempts to acquaint the student with the experimental approach to pharmacology and pharmacodynamics. It is designed to serve both as a laboratory outline and textbook on pharmacology.

Nederlandse Pharmacopee. Zesde Uitgave. Ministerie Van Sociale Zaken En Volksgezondheid, 1958. xxvii + 633 pp. 15.5 x 24 cm.

This new edition of the Nederlandse Pharmacopee represents a complete revision. The drugs for which specifications are provided are for the most part older therapeutic agents and pharmaceutical vehicles and necessities. Apparently no drugs restricted by patents have been included in this edition. This is marked contrast to the latest lists of admissions to the United States Pharmacopeia and the National Formulary.

Biochemical Preparations. vol. 6. Edited by Carl S. Vestling. John Wiley & Sons, Inc. New York, 1958. ix + 105 pp. 14.5 x 23 cm. Price $5.25.

Carefully checked procedures are presented for the preparation of crystalline animal cytochrome *c*, deoxyribonucleic acid, 2,3-diphosphoglyceric acid, *l*-α-glycerophosphorylcholine, 3-hydroxyanthranilic acid, β-hydroxy-β-methylglutaric acid, insulin, lanosterol from "isocholesterol," leucine aminopeptidase, α-methylserine and bis(hydroxymethyl) glycine, crystalline horse oxyhemoglobin, old yellow enzyme, crystalline papain and benzoyl-*l*-argininamide, crystalline muscle phosphorylase *a* and *b*, phosphoserine, ribonucleic acid from rat liver, ribulose diphosphate, and *dl*-tryptophan-7a-C^{14}.

Practical Clinical Biochemistry. 2nd ed. By HAROLD VARLEY. Interscience Publishers Inc., New York, 1958. viii + 635 pp. 14 x 21.5 cm. Price $6.50.

This book describes procedures of the many biochemical tests carried out in hospital laboratories as an aid to the diagnosis and treatment of disease. It is designed to be used primarily as a practical textbook.

Medizinal Kalender 1959. W. v. Brunn. Georg Thieme Verlag, Stuttgart, Germany, 1958. xx + 856 pp. 9 x 16 cm. Price DM 9.60. Distributed in the U. S. and Canada by Intercontinental Medical Book Corp., New York. Price $2.30.

A handbook of diagnoses and therapy of especial interest to physicians.

Kurzes Lehrbuch Der Pharmazeutischen Chemie, Fünfte Auflage. By K. BODENDORF. Springer-Verlag, Berlin W 35, Reichpietschufer 20, West Berlin, Germany, 1958. vii + 490 pp. 16.5 x 25 cm. Price DM 34.50.

Textbook of inorganic and organic chemistry designed for pharmacy and medical students.

Russian-English Medical Dictionary. By STANLEY JABLONSKI. Academic Press, Inc., New York, 1958. x + 423 pp. 15 x 23 cm. Price $11.

This dictionary is a feature of the Russian Scientific Translation Program conducted by the National Institutes of Health, U. S. Public Health Service. It is designed to assist in the translation of medical and allied books and papers written in the Russian language.

Simple Methods of Contraception. Edited by Winfield Best and Frederick S. Jaffe. Planned Parenthood Federation of America, New York, 1958. 64 pp. 15.5 x 23.5 cm.

Various simple contraceptive techniques are described and evaluated and their medical, social, and moral implications are discussed.

Chemical Transformations by Microorganisms. By FRANK H. STODOLA. John Wiley & Sons, Inc., New York, 1958. ix + 134 pp. 12.5 × 18.5 cm. Price $4.25.

This book is based on the second series of E. R. Squibb Lectures on Chemistry of Microbial Products, Rutgers University. The production of new and useful chemicals by microorganisms is discussed. A full account of the production of alpha-ketoglutaric acid and L-glutamic acid by fermentation is presented. How microorganisms are used in industry and recent information on research on mycobacterium tuberculosis are also included in the lectures.

Tuberkulose-Bucherei. Monographien zur Monatsschrift Der Tuberkulosearzt. Georg Thieme Verlag, Stuttgart, Germany, 1958. 152 pp. 16.5 x 24 cm. Price DM 17.80. Distributed in the United States by Intercontinental Medical Book Corporation, New York. Price $4.25.

A series of papers presented on the occasion of the dedication of the Robert Koch Tuberculosis Clinic of the University of Freiburg, in February 1958. The lectures cover the general field of tuberculostatic agents and the development of resistant strains of the tubercle bacillus.

Serological and Biochemical Comparisons of Proteins. Edited by William H. Cole. Rutgers University Press, New Brunswick, 1958. xi + 119 pp. 15.5 x 23.5 cm. Price $2.

Seven papers are reproduced in this paper-bound book which were presented during a program of studies on protein metabolism sponsored by the Bureau of Biological Research, Rutgers University, New Brunswick, N. J. The papers are designed to cover recent developments in the basic mechanisms that are concerned with protein interactions in the living organism.

The Guinea Pig in Research. By MARY ELIZABETH REID. Human Factors Research Bureau, Inc., Washington 4, D. C., 1958. 87 pp. 15 x 23 cm. Price $2.

This paper-bound booklet describes the known dietary needs of the guinea pig in quantitative terms together with a brief account of some aspects of its physiology which may be of interest to the nutritionist and may increase its usefulness in medical research.

A Contribution to the History of Ancient Transportation and Trade. By W. H. BLOME. Ann Arbor Publishers, Ann Arbor, 1958. vi + 86 pp. 21.5 x 28 cm. Paperbound. Printed by photo offset.

The author of this book presents data pertaining to ancient transportation, land routes of commerce, travel, transport by water, merchandise transported, with special emphasis on drugs, and the dangers and difficulties that were encountered. The author was formerly Chief Pharmacist with Frederick Stearns and Company and later Professor of Pharmacy at Wayne State University of Detroit. He is now living in retirement in Manchester, Mich.

Experimental Pharmacognosy. 2nd ed. By VARRO E. TYLER, Jr., and ARTHUR E. SCHWARTING. Burgess Publishing Co., Minneapolis, 1958. iii + 81 pp. 21 x 27 cm. Price $2.75. Paperbound. Printed by photo offset.

A laboratory outline designed to aid in teaching fundamental principles which are universally applicable. Biochemical processes are stressed, and a key for the identification of powdered drugs is included.

Vade-Mecum Du Vétérinaire. By A. BRION. VICOT Fréres, Editeurs, Paris, 1958. xv + 752 pp. 10.5 x 17.5 cm. Price 4,200 fr.

A presentation in capsule form of present day knowledge in most of the fields which interest the practicing veterinarian.

XVIIe Congres Des Sciences Pharmaceutiques Leiden, 1957. Conferences et Communications. D. B. Centen, Amsterdam, 1958. 351 pp. 16 x 24 cm. Price 195 fr.

A series of papers and discussions presented at the Seventeenth Congress of the Pharmaceutical Sciences held in Leiden in 1957. Most of the papers are written in French, German, or English, and the authors represent several nationalities.

Abrégé de Matière Medicale. Matières premieres d'origine vegetale. 3rd ed. By M. R. PARIS and H. MOYSE. Vigot Freres, Editeurs, Paris, 1958. 196 pp. 16 x 24 cm. Price 1,100 fr.

This book (in French) presents information in abstract form on material of vegetable origin used in materia medica. A classification of vegetable drugs according to their pharmacodynamic action is included and a subject index is appended.

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